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Clinical and Experimental

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ERYTHROPHAGOCYTOSIS: STANDARDIZATION OF A QUANTITATIVE TISSUE CULTURE TEST AND ITS APPLICATION TO HEMOLYTIC, MALIGNANT, AND INFECTIOUS DISEASES

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COLUMBUS, OHIO

THE study of varying peripheral factors influencing normal and abnormal red blood cell destruction has constituted the major effort in this laboratory in recent years. In a series of reports,[1-9] attention has been focused on a number of modifications of the surface of the erythrocyte in vitro and in vivo. In these studies the inadequacy of the classical in vitro laboratory procedures as an index of the destructive changes in vivo was soon evident. New in vitro methods having some counterpart to the in vivo processes were sought.

It was previously postulated that phagocytosis plays a paramount role in the mechanism for the actual removal of certain damaged or modified erythrocytes from the peripheral circulation. In order to examine this premise a method was established and standardized for detecting the susceptibility of human erythrocytes to phagocytosis by rabbit splenic macrophages grown in tissue culture; this is expressed as the *phagocytic index (P.I.)*. The content of this report is twofold: first, the description of the test itself, and second, the application of the test to 352 individuals in health and disease.

MATERIALS AND METHODS

I. *Tissue.*—The spleens of young domestic rabbits are aseptically removed under sodium pentobarbital anesthesia. The spleen is placed in a small amount of modified Earle's solution* (pH 7.4). The capsule is dissected away and the tissue placed on a Maximow depression slide† and minced into pieces approximately 1 mm. in diameter with sharp scissors. The splenic tissue is rinsed in three changes of the Earle's solution. Rabbits fasted overnight are the source of tissue or serum.

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*The modified Earle's solution is prepared as follows: To 1000 ml. of redistilled water, the following are added and dissolved separately, in the order listed:

NaCl	6.80 Gm.
KCl	0.40 Gm.
$CaCl_2$ anhydrous	0.20 Gm.
$MgSO_4 \cdot 7H_2O$	0.10 Gm.
$NaH_2PO_4 \cdot H_2O$	0.125 Gm.
Glucose	1.00 Gm.

Dissolve 2.20 Gm. of $NaHCO_3$ in 100 ml. of redistilled water in a second flask. Autoclave both flasks, then adjust pH of first flask to 7.4 by addition of a small amount of $NaHCO_3$ solution (approximately 10 ml.). The solution should be adjusted to proper pH immediately before use, since the pH of the solution rises upon storage.

†Maximow depression slides, round depression, 3 by 1¾ inches, are obtainable from Clay Adams, Inc., 141 E. 25th Street, New York, N. Y.

II. *Nutrient Medium.*—The nutrient medium consists of a mixture of 40 parts fresh rabbit serum, either pooled or autologous, and 60 parts of Earle's solution (pH 7.4). Penicillin G potassium and dihydrostreptomycin are added in the final concentration of 100 units of each per milliliter of nutrient medium.

III. *Cover Slip Cultures.*—The method used is a modification of the cover slip method described by Cameron[10] and Parker.[11] Scrupulously clean sterile glassware is used routinely. Glass rings 3 mm. wide with an outside diameter of 20 mm. are dipped in sterile melted paraffin and placed on round ⅞ inch No. 1 cover slips. A drop of Earle's solution containing several explants is placed in the center of each cover slip using a capillary pipette. The excess fluid is drawn off. The cultures are allowed to dry at room temperature for three to five minutes until the fluid has evaporated. Two to three drops of the nutrient medium are then added. An ordinary 1 inch by 3 inch microscope slide is warmed over an alcohol lamp and placed on the ring, the melted paraffin giving a temporary seal (Fig. 1). A 4:1 paraffin-vaseline mixture is used to give a complete seal. The cultures are incubated at 37° C., slide up, for forty-eight hours, after which they are inspected under low power. The rings and slides of those cultures, which are to be kept, are separated with a spatula and the fluid removed with a sterile capillary pipette. Three drops of *freshly prepared* nutrient medium are then added, the cultures resealed and incubated another twenty-four hours before use.

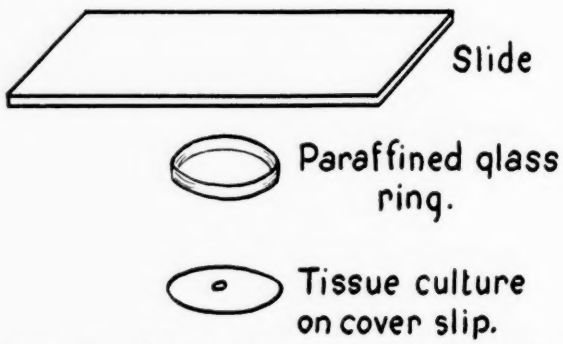

Fig. 1.—Diagram of the parts of the tissue culture system in the incubating position. The preparation is inverted for microscopic examination.

IV. *Test Procedure.*—

A. Erythrocytes: Whole blood from human beings is obtained by venipuncture and from rabbits by cardiac puncture. Balanced oxalate is used as the anticoagulant. The erythrocytes are washed three times with phosphate buffered saline (pH 7.4) and are then diluted with Earle's solution to a 0.3 per cent suspension.

B. Serum: A mixture of 80 parts rabbit serum, stored for at least eight days at 5° C., and 20 parts of Earle's solution is used for all routine tests.

C. Technique: The cultures are reopened as before and the nutrient fluid drawn off. One drop each of cell suspension and serum is added, after which the culture is resealed and incubated for thirty minutes. Examination for phagocytosis is then made with the preparation inverted, using 430× magnification.

RESULTS

I. *Standardization of Test and Phagocytic Index.*—When three- to five-day-old cultures of rabbit spleens, prepared as described, were observed before the addition of the test cells, large numbers of living macrophages could be observed growing out from the explant on the cover slip. The number of such cells and their viability varied in a large series of such cultures, but it soon be-

came possible to obtain satisfactory cultures in 75 to 90 per cent of those attempted. Identification of the nonviable macrophages was made by changes occurring in the cytoplasm of the cell, granulation, lack of motility, and the development of a spherical shape. This enhanced the efficiency of the test since these cells were not counted in a test system and cultures containing numerous nonviable cells were discarded.

Since some of these macrophages already contained easily identifiable erythrocytes from the rabbit from which they were obtained, the number of these present in a total of fifty macrophages served as a base line to be subtracted from the count obtained after addition of the test cells (Fig. 2). When a suitable base line count had been made, the fluid of the culture was removed and fresh fluid added along with the test erythrocytes. The cultures were re-

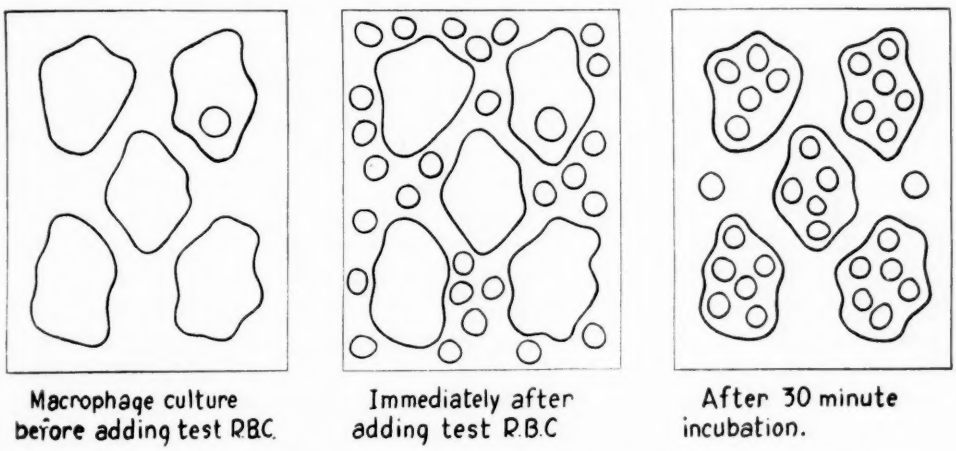

Fig. 2.—Three- to five-day-old tissue cultures of rabbit spleens have approximately a 98 per cent macrophage population. There may be a few macrophages containing easily identifiable R.B.C. from the rabbit, whose spleen was the tissue source; these are the base line macrophages and are subtracted from the final reading. The test R.B.C. are incubated with the macrophage system for thirty minutes. Final reading of the percentage of macrophages containing R.B.C. (minus the base line) is the *phagocytic index (P.I.)*.

placed in the incubator and removed only for counting. A total of fifty macrophages per culture were inspected for: (1) the number of macrophages containing red cells; and, (2) the number of red cells ingested. The base line counts were subtracted and the results finally expressed as the percentage of macrophages showing phagocytosis of the test cells. This was selected as the standard *phagocytic index (P.I.)* since this type of count was found to be more consistent and accurate.

Standardization was made from numerous tests on normal rabbit and human erythrocytes and on both types of cells which had been modified previously by sensitization with specific antibody, or by various other procedures as treatment with enzymes, tuberculin, and virus. In a large series of such tests it was found that phagocytosis in most instances was maximum in twenty to thirty-five minutes after the addition of the test cell, so that the *phagocytic index (P.I.)* was computed after thirty minutes' incubation. The various observations on

phagocytosis by macrophages in tissue culture and the effects of in vitro modifications of the cells and serum will be made in a later report.

Phagocytosis of Rabbit Erythrocytes.—The P.I. values of the cells of normal rabbits were within a range of 3 to 7 per cent. At first, the serum of the same rabbit was used, but later tests using serum from other rabbits indicated this was not a factor using normal erythrocytes. Various factors, such as sensitization and trypsinization of rabbit cells in vitro increased the phagocytic index variously from 30 to 92 per cent.

Phagocytosis of Human Erythrocytes.—It was assumed originally that human cells would have a higher P.I. in cultures of rabbit macrophages because of the species difference and the opsonizing action of the normal rabbit serum. However, normal human cells were *not* phagocytized to any greater degree than rabbit cells either in the presence of rabbit or human serum. Further studies to be reported demonstrated that in vitro modifications were necessary to increase phagocytosis. This confirms the observation of Bloom[12] that pigeon cells were not taken up by rabbit lung macrophages unless the red cells were sensitized. Thus, because of this lack of species specificity and the failure of rabbit serum to opsonize human cells, it was possible to use the same system of rabbit serum—macrophage cultures for testing human cells. At the same time, experiments indicated that modifications of human cells would increase the P.I. This made it possible to detect similar effects produced on cells in diseased individuals. The use of the rabbit serum also avoided a variable and still unknown factor in normal human serum which was shown to inhibit the opsonizing effect of substances like trypsin in vitro. It was found that hemolysis of certain modified human erythrocytes occurred in the tissue culture if *fresh* normal rabbit serum was present. For this reason rabbit serum, stored under sterile conditions for a week at refrigerator temperature, was used routinely.

It is noted in Fig. 3 and in Table I that the red blood cells of 59 normal individuals were phagocytized within a fairly limited range of P.I. values (from a −8 to +12 per cent*). Individuals of both sexes in age from infancy to 70 years were included. The P.I. values of this group are consistent with the hypothesis that measurements from this group are normally distributed with mean 2.9 per cent and standard deviation 4.1.

II. *The Phagocytic Index in Disease.*—Early in the course of the examination of the above group several instances occurred which suggested that the P.I. was markedly increased by the presence of certain diseases. This prompted the application of the test to the groups of diseased individuals listed in Fig. 3 and in Table I. For example, the first group of 14 individuals chosen as normal subjects was composed of medical students seemingly in good health. Short histories and specimens were taken and tests performed by independent teams. It was noted that of the 14, the two individuals whose cells showed elevated P.I. values (21 and 25), one complained of a sore throat of several weeks' duration and the other had recently had what was diagnosed as a viruslike infection involving the gastrointestinal tract.

*The negative values are due to a greater base line value than was obtained after incubation with the test cells.

SCATTER DIAGRAM OF PHAGOCYTIC INDICES

SCATTER DIAGRAM OF PHAGOCYTIC INDICES IN NORMAL AND DISEASE STATES.

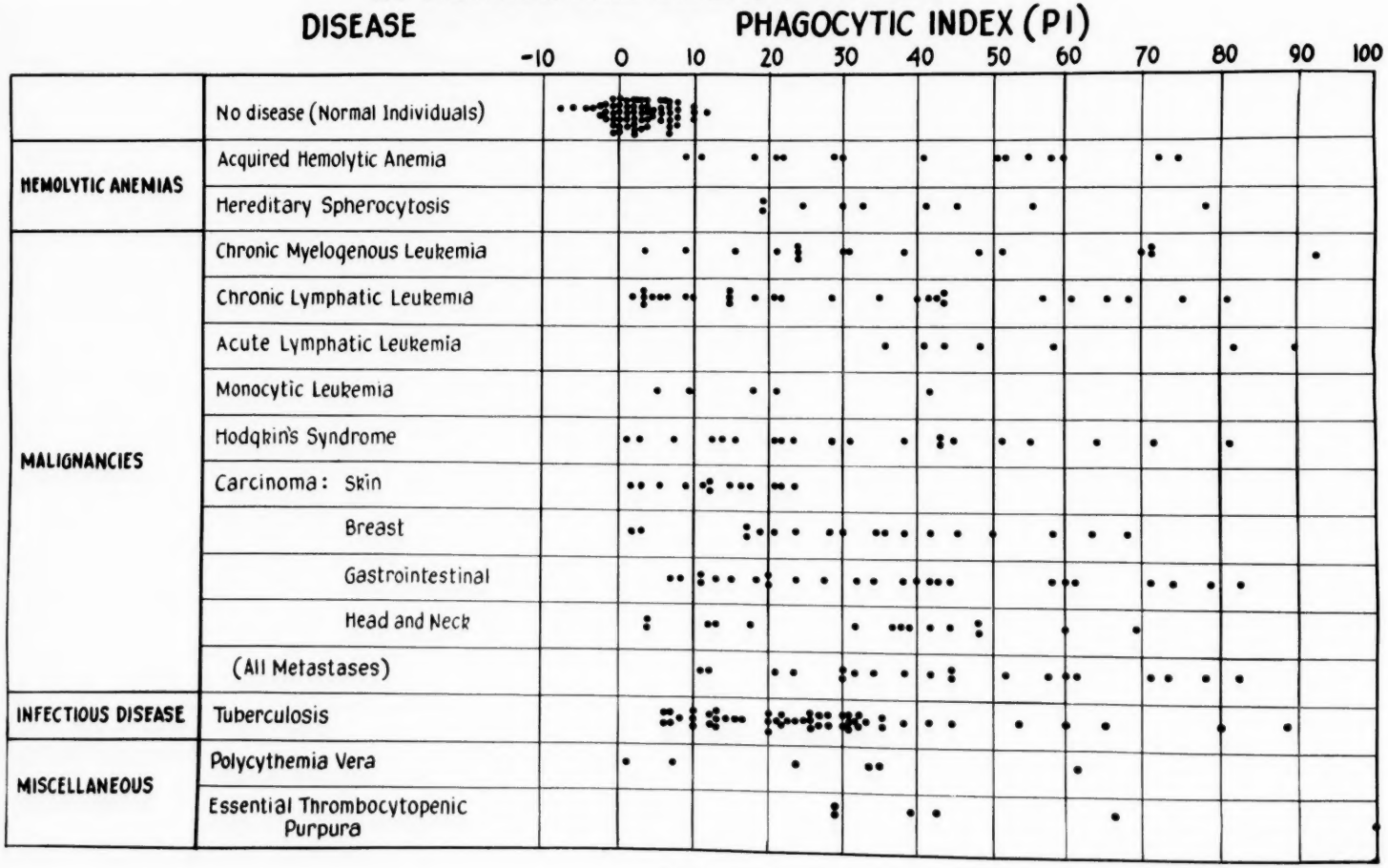

Fig. 3.

TABLE I

DISEASE	NUMBER OF INDIVIDUALS MEASURED	PER CENT OF MACROPHAGES CONTAINING RED BLOOD CELLS	MEDIAN
No Disease (normal)	59	12,10,10,10,8,8,8,8, 7,7,7,7,7,6,6,6,6,5, 5,4,4,4,4,4,4,3,3.3, 3,3,2,2,2,2,2,2,1.1, 1,1,1,0,0,0,0,0,0,0, 0,-1,-1,-1,-2,-2,-2, -3,-4,-6,-8	3.0
Hemolytic Anemias:			
Acquired hemolytic anemia	16	74,72,60,58 55,52,51, 41,30,28,28,22,21,18, 11,9	35.5
Hereditary spherocytosis	7	78,56,46,41,33,30.25, 19,19	33.0
Erythroblastosis	2	39,17	
Sickle-cell anemia	2	12,4	
Thalassemia major	1	5	
Malignancies:			
Chronic myelogenous leukemia	16	93,72,72,70,52,48,38, 31,30,24,24,24,21,16, 9,4	30.5
Chronic lymphatic leukemia	26	81,76,68,66,61,57,44, 44,43,42,40,35,28,22, 21,18,15,15,15,10,9 7,6,5,4,4,4,2	21.5
Acute lymphatic leukemia	7	90,82,59,48,44,41,36	48.0
Monocytic leukemia	5	42,21,18,9,6	18.0
Hodgkin's syndrome	20	81,72,64,56,52,46,44 44,38,31.28,24,22,21, 16,14,13,7,3,1	29.5
Carcinoma:			
Skin	13	24,22.21,17,16,15,12, 12,11,9,6,4,2	12.0
Breast	18	68,64,58,50,46,42,38, 36,35,30,28,24,21,19, 17,17,3,2	32.5
Gastrointestinal	25	83,78,74,71,62,60,58, 45,43,42,40,38,34,32, 27,24,20.20,18,15,13, 11,11,8,7	34.0
Head and neck	15	69,60,48,47.45,42,39, 38,36,32,17,14.13.4 4	38.0
All-metastases	20	83,78,74,71,62,60,58, 52,45,45,42,38,35,32, 30,30,24,21,12,11	43.5
Lymphosarcoma	4	84,58,38,24	
Reticulum-cell sarcoma	3	40,31,9	
Eosinophilic granuloma	1	54	
Infectious Diseases:			
Tuberculosis	49	88,80,66,60,54,45,42, 38,36,36,34,33,33.32, 31,31,31,30,30,27,27, 26,26,26,25,24,23,22, 22,21,21,21,21,17,16, 15,14,14,14,13,13,10, 10,10,8,7,7,6,6	25.0
Tuberculous meningitis	2	34,17	
Gas gangrene	2	21,16	
Tetanus	1	32	
Brucellosis	1	19	
Streptococcal infection	1	17	
Infectious mononucleosis	2	38,24	
Hepatitis	1	54	
Acute rheumatic fever	1	38	
Undiagnosed	4	52,46,42,24	

TABLE I—CONT'D

Miscellaneous:			
Essential thrombocytopenic purpura	6	100,67,43,39,29,29	41.0
Polycythemia vera	6	62,35,34,24,7,2.	29.0
Nutritional amenia	4	46,38,17,0	
Thrombophlebitis	1	7	
Aplastic anemia	3	6,3,1	
Reticuloendotheliosis	1	2	
Banti's syndrome	2	52,20	
Neutropenia	3	67,28,19	
Felty's syndrome	1	60	

Further suggestion along this line came from the findings of repeated tests on the cells of laboratory workers. Individuals whose cells had given P.I. values in the normal range in three or four weekly tests suddenly showed marked increase coincident with development of symptoms. In the first of these observations, blood cells obtained the day the initial symptoms of a common cold occurred showed a P.I. of 30, as compared to previous readings while in good health of 0 and 1. Similar findings occurred in four other individuals who prior to the development of symptoms showed P.I. values of 2,3,4, and 1, respectively. Subsequent tests over a time period including the illness and recovery gave the following P.I. values for each: 29,40,48,16,0; 60,52,58,17,3; 36,12; and 24,14. In the two individuals where the highest values were 48 and 60, the P.I. returned to normal in six weeks to two months.

There were four groups of diseases to which application of the phagocytic index was made. These are listed in Table I. Logically, the hemolytic anemias were the first of the clinical entities studied. It may be noted that in sixteen of the acquired hemolytic anemias, all except two were greater than the maximum value (12) shown by any normal individual. In all of the seven cases of hereditary spherocytosis the P.I. was elevated. In only two was the spleen intact (P.I. = 41,78). One erythroblastotic infant tested the day after delivery had a P.I. of 39; in another one-week-old infant the P.I. was 17. Neither the single thalassemia major nor the two sickle-cell anemias were appreciably elevated.

In the miscellaneous group it was noted that all of the six essential thrombocytopenic purpuras had elevated P.I. values. This is of particular interest in view of the recent work of Harrington and associates[13] on the immunologic abnormalities existing in these states. Essential thrombocytopenic purpura and certain of the hemolytic anemias seem to have many factors in common. Other entities which at times show varying degrees of excessive peripheral destruction—splenic neutropenia, Felty's syndrome, Banti's syndrome—all showed elevated indices.

The chance observation that the P.I. was elevated during a minor infection as coryza led to more extensive application in a series of infectious diseases. The recent observations of Moolten and Clark[18-20] on the relation of hemolytic anemia and virus infection intensified interest in this direction. The largest single group of these was forty-nine cases of tuberculosis with a median P.I. of 25. All except eight of these cases were larger than the maximum value (12) shown by any normal individual. In two cases of tuberculous

meningitis values were increased. P.I. values were also elevated in gas gangrene, tetanus, brucellosis, streptococcal infection, infectious mononucleosis, hepatitis, acute rheumatic fever, and in four undiagnosed cases but tentatively categorized as infectious diseases.

The recent interest of the short-lived erythrocyte in many instances of malignant disease[14-17] suggested investigation of erythrophagocytosis in this group. The range of values found was from 2 per cent to 93 per cent. The thirteen cases of skin cancer, with a median 12 per cent, appear to have the lowest phagocytic indices. The twenty malignancies having metastases had a median of 43.5 per cent.

A statistical test was carried out for each diseased group to test the hypothesis that the P.I. values for the diseased and normal groups are equal. The alternative was that the P.I. values for the diseased group are greater. Comparisons were made using the U test of significance.[21] Small values of U are consistent with the alternative. If the hypotheses were correct the probability of obtaining a U as small as the one observed was, in all comparisons, less than 0.001. One may infer that the per cent phagocytosis is larger for the diseases tested than for the normal group.

DISCUSSION

(1) The macrophage, (2) the surrounding medium, and (3) the cell phagocytized have relative roles in influencing phagocytosis. It has been long recognized that this mechanism plays a significant role in various types of clinical hemolytic anemia. More and more attention has been directed to the erythrocyte, per se, as the most important of the three phases involved. This study was undertaken to test the susceptibility of circulating erythrocytes in health and disease to phagocytosis. The macrophage and the surrounding medium were controlled. The variable was the erythrocyte and its susceptibility to ingestion and digestion by the macrophage.

This test has been standardized and applied to four groups of clinical diseases. The statistical evaluation of the quantitative differences in these diseased individuals compared with normal persons seems to support the belief that the test detects changes in red cells which make them susceptible to phagocytosis and that erythrocyte modifying factors of varying kinds are at work in disease. The presence of normal P.I. values in certain diseased persons may indicate that the number and nature of the erythrocyte modifying factors and their imprint on the cell, as measured by phagocytosis, may vary in time and intensity during the course of disease. The effects of some changes may even persist temporarily on recovery. Furthermore, stabilizing influences which have been minimized or removed in this test may operate in vivo. For example, it appears that some modifications may be minimized in the presence of normal serum. It seems reasonable, however, that certain of these factors do operate in disease and that the changes produced *can* lead to removal by phagocytosis. On the other hand, the more or less constant presence of a small percentage of phagocytable cells in normal individuals suggests that any further correlation between the P.I. and the shortened survival of

erythrocytes in hemolytic processes will require more knowledge of the modifying factors and the in vivo forces tending to stabilize the cells.

Obviously, the next steps are to determine the nature of these modifying and stabilizing factors, and their relationship to the presence of such abnormal hematologic findings in hemolytic anemia as morphologic characteristics, increased osmotic and mechanical fragilities, changes in the electrophoretic mobility, the presence of antibody, disarrangement of the enzyme equilibrium, and shortened red-cell survival. Some of these factors have already been detected and further work is in progress which will be the subject of future reports.

SUMMARY

The primary elements influencing phagocytosis are (1) the macrophage, (2) the surrounding medium, and (3) the phagocytized cell. Erythrophagocytosis of human cells by rabbit macrophages in tissue culture has been measured by a test procedure standardized to detect changes in the red cell. The range of the *phagocytic index (P.I.)* values for erythrocytes of 59 normal individuals has been established, and the effect of certain disease states on the P.I. has been studied on 293 patients. These included hemolytic anemias, malignant diseases, and infectious diseases. It is concluded that there exist significant quantitative differences in *phagocytic index* values of the erythrocytes of individuals in these disease groups and in normal persons.

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PERFORMANCE OF A SODIUM IODIDE SCINTILLATION COUNTER IN MEASUREMENT OF I^{131} UPTAKE BY THE THYROID

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As a scintillating phosphor, thallium-activated sodium iodide is well known to possess a high sensitivity to gamma radiation. A disadvantage of this compound is its hygroscopic property. Immersion in oil or application of a plastic coating does not prevent a progressive loss of transparency accompanied by yellow discoloration. The recent introduction of moisture-proof "canned" crystals* has made possible the construction of stable counting devices for use in clinical procedures where gamma-emitting isotopes are employed. When radioactivity is measured "in vivo" a very sensitive counter permits restriction of dosage to the lowest possible level, and compensates for the attenuating effect of remote placement or collimation when the technique necessitates these modifications. The behavior of a sodium iodide scintillation counter will be described. Emphasis is directed toward its performance in the measurement of the uptake of I^{131} by the thyroid.

CONSTRUCTION

The essential components of the counter are illustrated in Fig. 1. The detecting crystal measures 1½ inches in diameter by 1 inch in thickness. Placement over the thyroid in correct alignment is accomplished by a built-in adjustable spacing rod (Fig. 2). Flexibility of positioning is facilitated by attachment of the counter to the folding arm of a dental x-ray unit mounted to the central column of a mobile x-ray stand which is itself bolted to the wall.

Interchangeable collimators, constructed of type metal rather than pure lead to provide maximum resistance to accidental impact, range from ½ inch to 1½ inch inside diameter and from 1 inch to 4 inches in length. The long narrow types are useful in close-range measurement of differential uptake by thyroid nodules. Larger openings are applicable for total thyroid uptake and for brain tumor localization. Short broad types placed next to the skin are useful for studies of peripheral blood flow. For radiocardiograms, collimators may be omitted entirely.

SENSITIVITY TO I^{131} AND BACKGROUND

The sensitivity of the counter to gamma radiation is indeed remarkable. A carefully assayed† spot source of I^{131} placed at 100 cm. from the face of the

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*Obtained through Harshaw Chemical Co., 1945 E. 97th Street, Cleveland, Ohio.

†The reference material, previously checked on several occasions against NBS primary standards of I^{131}, was an NBS radium D plus E source. The technique of comparison was that of absorption-extrapolation.

crystal, without a collimator in place and filtered with 138 mg. per square centimeter of aluminum in addition to that which protected the crystal, gave 162 counts per minute per microcurie. With another experimental crystal which had acquired a slight yellow hue due to its having been imperfectly sealed for a year in a temporary container, the count was 148. In that the solid angle subtended by a 1½ inch circle at 100 cm. would allow 201 gamma photons per microcurie per minute to reach the surface of the crystal,† the calculated efficiency of count for the better of the two crystals would be 80 per cent.

Efficiency, of course, depends upon a combination of stopping power and the capacity of the crystals to produce light pulses of sufficient intensity to be recorded. With a crystal one inch thick, the absorbing power due to photo-electric effect and compton scatter may be calculated on the basis of the absorption coefficients of NaI for the gamma components of I^{131}. The value so

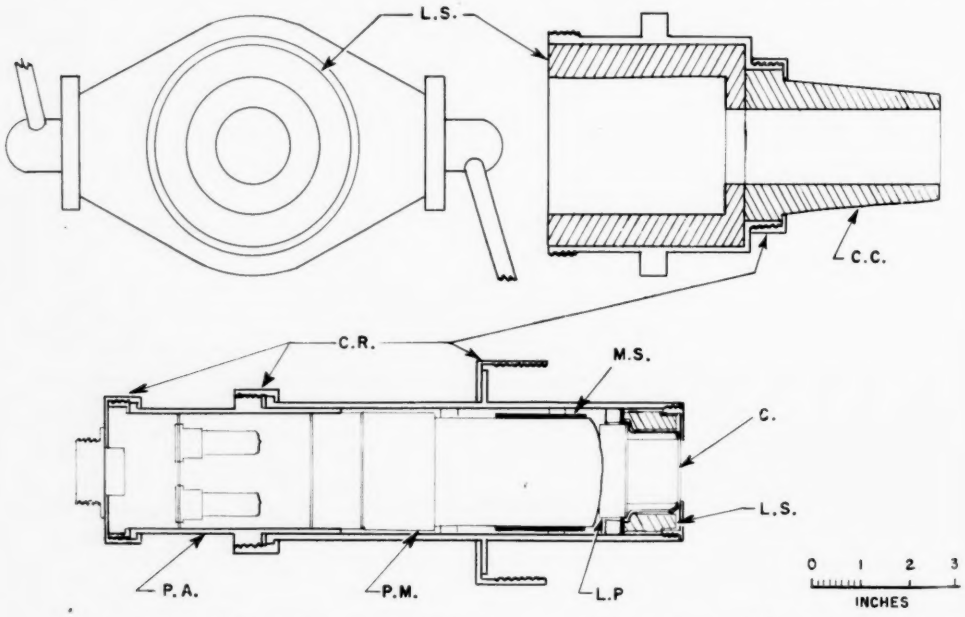

Fig. 1.—*L.S.*, lead shield; *C.C.*, collimator; *C.R.*, coupling rings; *M.S.*, magnetic shield (soft iron); *C.*, crystal; *P.A.*, Preamplifier (amplification factor, 200/1); *P.M.*, RCA 5819 photomultiplier tube; *L.P.*, lucite light pipe. The counter functions well with an SC-1 autoscaler, which was used in the present study, but cannot be operated with all types of scalers.

obtained approximates 73 per cent. In order to determine this value by actual experiment, a similar crystal of NaI was interposed between a source and the counter to test its attenuating effect on the count. The spot source was located at 50 cm. from the center of count, and a lead collimator one inch in bore by 4 inches in length was placed between the absorbing crystal and the front of the counter. The observed absorption was 72 per cent. It is thus

†This assumes one gamma photon per disintegration. With a small solid angle the two gamma photons in cascade result in a 6 per cent excess; however, there is a comparable loss from internal conversion.

apparent that the efficiency of count exceeds the value for per cent absorption determined both by calculation and by experimental measurement. This would mean not only that a very high proportion of light pulses are sufficiently strong to be detected by the phototube, but also that extra pulses have somewhere arisen. To be considered are afterpulses within the phototube, scatter arising either from unabsorbed radiation interacting with objects beyond or in front of the crystal, and delayed phosphorescence within the crystal. That the latter phenomenon can occur with thallium-activated potassium iodide has already been demonstrated.[1]

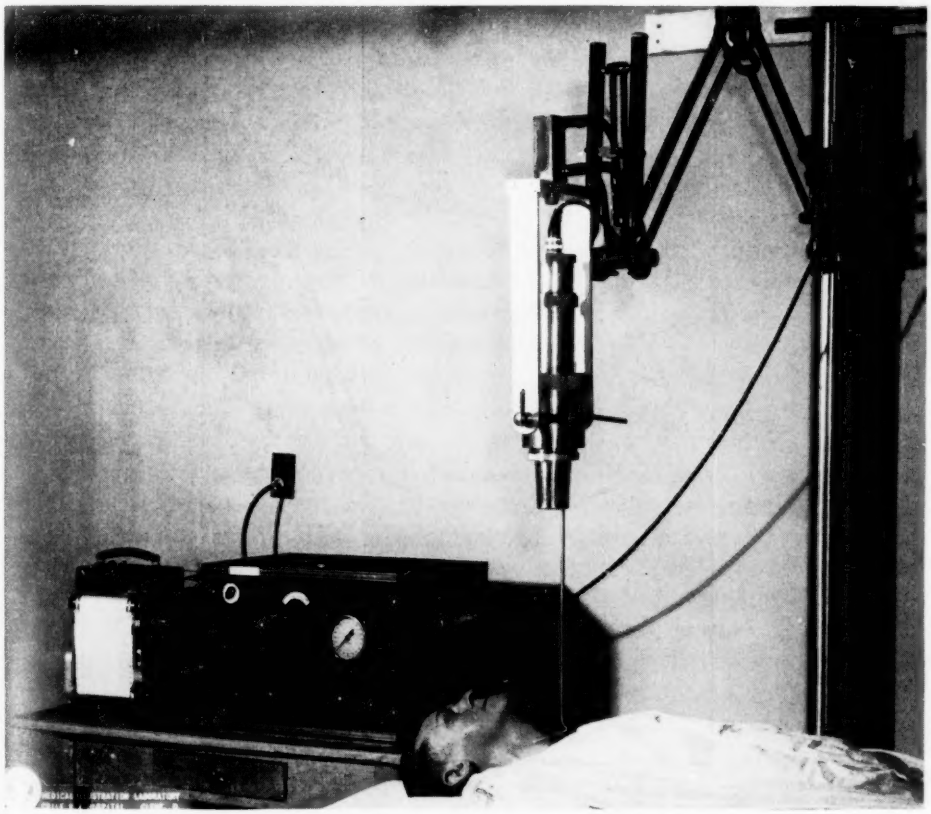

Fig. 2.—Measurement of thyroid uptake.

Unshielded, the counter is very sensitive to background radiation; however, minimal shielding reduces the count substantially. In a typical experiment, the background value of the entirely unshielded instrument when located in the laboratory was 8,000 counts per minute. One-sixteenth inch of lead around the forepart of the housing and in front of the crystal reduced the figure to 1,900, one-eighth inch 1,300, one inch 430, two inches 280, and three inches 190. When the instrument was placed in a remote room 200 feet from

the laboratory the background was 4,100 unshielded and was reduced to 1,033, 1,100, and 410 with one-sixteenth, one-eighth, and one inch of lead, respectively. It is apparent that a very soft component of background radiation contributes to the high count in the unshielded state. For clinical purposes it is not necessary to use more than one-half to three-fourths inch of lead as a shield. With the five-eighths inch envelope used in the present instrument, the background in the laboratory is usually 2,000 counts per minute if the front of the crystal is uncollimated and 550 when the 1½ by 4 inch collimator is in place.

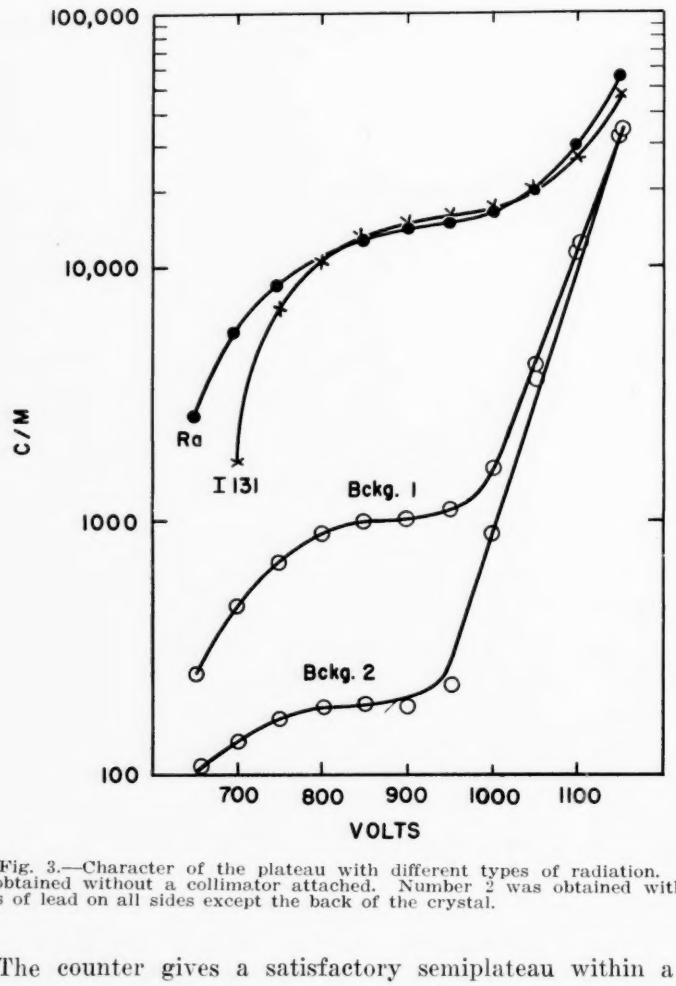

Fig. 3.—Character of the plateau with different types of radiation. Background No. 1 was obtained without a collimator attached. Number 2 was obtained with a shield of three inches of lead on all sides except the back of the crystal.

The counter gives a satisfactory semiplateau within a 100-volt range (Fig. 3). Photomultiplier tubes vary widely in the minimum voltage which produces noise. This is of little consequence, however, because the threshold voltage for counting and the lateral position of the plateau are correspondingly shifted. With the input sensitivity and preamplification of the present equip-

ment, the slope of the plateau for radium is similar to that of I^{131}. Thus a permanent radium standard may be employed for purposes of reference and control.

A crystal one-half inch thick by one inch in diameter was tested in place of the larger one. With 10 microcuries at 100 cm. and the counter uncollimated the net count was 480 per minute as compared to 1,620 with the larger crystal. The corresponding background counts were 313 and 1,330. Calculation of the per cent of incident gamma radiation counted by the one-half inch crystal gave a value of 54 per cent. The slight disadvantage of background count ratio with the larger crystal is outweighed by the considerable advantage of a much higher count. Of course the background count ratio suffers more seriously if very narrow collimation is used with the wider crystal; however, this is not of great consequence unless very low activities are being counted. A crystal thicker than one inch would not add sufficiently to the count to offset the expected increment in background along with somewhat poorer light transmission to the phototube.

THYROID UPTAKE

Thyroid uptake is commonly estimated by comparison of a count obtained over the gland with the count of a standard solution of I^{131} placed at the same distance from the counter. Certain precautions are necessary in order to insure that the count over the neck is a reasonably true measure of the actual thyroid content. Chief sources of error are radioactivity in surrounding tissues, scatter, and inaccuracies of estimation of distance to the center of radiation.

Extrathyroidal Radioactivity.—The amount of radioiodine in surrounding tissues is of little consequence after twenty-four hours, but during the first several hours after the dose is administered, radiation from the body as a whole is of sufficient magnitude to necessitate either collimation of the shielded counter, or adequate shielding of the patient's body over regions beyond the immediate vicinity of the gland. The former procedure is more practical and has proved to be very satisfactory for routine procedures involving one-hour uptake, collection rate, or plasma clearance. Subtraction of a count over the lower thigh serves to correct for the component of activity present in extrathyroid tissue of the neck.

A collimator with a one and one-half inch bore and four inches in length has been found suitable. At 50 cm. from the crystal, this collimator restricts the total field to an area 30 cm. in diameter. A spot source is counted with an efficiency above 90 per cent when it lies within a circle 8 cm. in diameter. An extended circular source 10 cm. in diameter gives a count 92 per cent of that of a 1 cm. spot source in line with the central axis.

Scatter.—A certain proportion of gamma radiation emanating from the thyroid, which is initially directed away from the counting device, will interact with the surrounding tissue so as to produce secondary compton photons of somewhat lower energy. Many of these rays are redirected toward the

counter and thereby can cause an increase in count above that which would be observed if the thyroid gland were suspended freely in air. One way to correct for the erroneously high count of the gland in situ is to count the standard under conditions simulating the situation in the patient's neck. A simple technique is to immerse a 50 ml. flask containing the standard in a one liter beaker of water next to the edge near the counter. Such an arrangement used in conjunction with the present counter leads to a 27 per cent higher count than when the flask is suspended in air. An alternate procedure is to place a source in the neck of a cadaver or in the esophagus of a living subject and from the increase so observed to calculate a correction factor.[2] With either procedure it must be assumed that variations in the size of the patient's neck and of the gland itself will not seriously influence the amount of secondary scatter.

In order to test this assumption, the effect of the size and extent of backing material was studied. From the first line of Table I, it is apparent that the per cent increase due to scatter was twice as large when the backing consisted of a 15-inch cylinder of water than with a one-liter beaker. The effect of a subject's neck was intermediate. Of interest is the fact that a lead brick gave less augmentation than did water or tissue. This is undoubtedly due to the relatively smaller proportion of compton scatter from an element of high atomic number.

TABLE I. MAGNITUDE OF SCATTER WITH NaI COUNTER
(Net counts per minute; 50 microcuries I^{131} at 50 cm.)

FILTER	IN AIR	BACKED BY WATER IN 1,000 ML. BEAKER	ON PATIENT'S NECK	BACKED BY 2 INCH LEAD	BACKED BY 15 INCH CYLINDER OF WATER
0	28,000	35,700 (24%)	39,500 (37%)	33,700 (17%)	45,500 (58%)
$\frac{1}{32}$ inch lead	19,700	21,300 (8%)			
$\frac{1}{16}$ inch lead	15,000	15,200 (1½%)			15,900 (6%)
$\frac{1}{8}$ inch lead	9,200	9,200			9,300 (1%)

A spot source on scotch tape was suspended in an open area of the room for the "air" reading and attached to the front of the backing object for detection of increase due to scatter. Collimation was such that all points within 10 cm. of the axis counted within 5 per cent of that at the center of the axis.

That a considerable element of "diagonally derived scatter" is included along with straight backscatter was shown by placement of two-inch lead bricks in front of the 15-inch cylinder of water so as to restrict all scattered radiation reaching the counter to that from an orifice four inches square centered around the spot source. With this arrangement, the increase from scatter was only half as great as when the full area of water was in view.

It has been shown (with other types of counters) that the softer scattered rays may be effectively eliminated by the interposition of a lead filter in front of the counter.[3, 4] Confirmation of this effect with the present counter is recorded in Table II. One-sixteenth inch of lead serves to remove nearly all of the softer scattered rays while passing two-thirds of the primary radiation. If a variable discriminator setting were available in the scaling system it should be possible to achieve the same result, and perhaps with less attenua-

TABLE II. COMPARISON OF SCATTER WITH VARIOUS COUNTING DEVICES
(50 microcuries I^{131} at 50 cm.; not less than 5,000 total counts)

		NET COUNTS PER MINUTE			
	FILTER	AIR	15 INCH WATER	% INCREASE OVER AIR	BACKGROUND
Geiger tube Copper cathode 7N5 Victoreen	0	201	231	15	62
	1/8" Pb	84	86	—	41
Anthracene Crystal	0	1,160	1,140	—	96
	1/8" Pb	530	540	—	46
NaI Crystal	0	28,800	45,500	58	830
	1/8" Pb	8,000	8,080	—	590

The Geiger tube was fitted with a 170 mg. per square centimeter layer of lead foil in front of the mica window for reinforcement of gamma sensitivity. The Anthracene scintillation counter was identical with that described by MacIntyre.[5]

tion of count derived from primary radiation, by increasing the discrimination level so as to eliminate the weaker pulses arising from the soft scattered radiation.

In order to determine the magnitude and variation of error due to scatter in actual subjects, counts of the thyroid and of a standard contained in a 50 ml. flask were made both with and without a one-sixteenth inch lead filter in a series of 25 subjects. Under such circumstances, the increase in calculated uptake when the filter was not used represented the augmentation resulting from scatter. The mean increase was 25 per cent (standard error of mean, 2; range, 14 to 46 per cent). In general, the lower values were obtained in thin subjects, while the highest readings encountered were in subjects with very thick necks. It, therefore, is apparent that the variability of scatter is quite significant, and that it is preferable to eliminate it by filtration rather than to attempt to correct for it.

During the past several years in this laboratory several types of counters have been employed for thyroid uptake measurements. It has now become apparent that there is a wide variation in sensitivity of various counters to scattered radiation. From the data in Table II, it is obvious that the sodium iodide scintillation counter is extremely sensitive and therefore requires special precautions in order to prevent the spuriously high count when thyroid uptake is being measured.

Distance of Gland.—Because of the necessity of short-range placement when counters of low sensitivity are used, the error due to unknown variations in the depth of the center of radiation, as with large deep-lying glands, may exceed a reasonable tolerance of accuracy. Attempts to avoid such errors of geometry have been made by the employment of four separate counters encircling the neck and connected in parallel to a single scaler.[6] The reliability of this method has been criticised.[7] Less ponderous, but still requiring careful spacing and fairly precise counting is the method of making readings at two or more distances from the neck and applying the inverse square law to determine the location of the true center of radiation within the gland.[2, 4] However, it is apparent that if the distance of the counter from the surface of the neck is sufficiently great, the error due to a variable depth of the center of

radiation will be minimized so that special corrective measures may be dispensed with in the great majority of patients, and a single count from the anterior surface of the neck should suffice.

The sodium iodide counter is sufficiently sensitive so that counts may routinely be made at a distance of 50 cm. Even with the one-sixteenth inch lead filter in place, 25 microcuries of I^{131} gives 7,500 net counts per minute (12 times background). At this distance, one would anticipate no more than a 10 per cent error due to a deep-lying center of radiation. In order to test this assumption on a mock-up model, counts were made of I^{131} samples contained in volumes varying from 10 to 250 ml. and placed at 50 cm. between the near edge of the volumetric flask and the counter. The one-sixteenth inch lead filter was included. The diminution of count when the 250 ml. flask was substituted for one of 50 ml. capacity was only 10 per cent, while the 25 ml. flask gave a value 5 per cent higher than the 50. With a great majority of patients, therefore, it would not seem necessary to determine the exact location of the center of radiation by dual or multiple counts at varying distances. In routine practice, one reading is made at 50 cm. from the surface of the thyroid isthmus and another at 50 cm. from the surface of a 50 ml. volumetric flask containing the standard. Only in the case of large, deep-lying glands is it considered necessary to make readings at two or more distances in order to calculate the true center of radiation. The present counter with the one and one-half by four inch collimator in place follows the inverse square law to a distance as close as 15 cm. both with a spot source and with spherical volumes varying from 10 to 100 ml.

It is questionable whether an attempt to correct for the 3 per cent per centimeter absorption by the tissue which intervenes between the surface of the neck and a derived center of radiation is either necessary or practical. In instances where the center of activity is calculated to lie rather far beyond the external surface of the cricoid, the remote position may sometimes be due to an active nodule lying in a posterolateral position rather than to a gland which is deeply buried beneath overlying structures. When distance to the standard is measured from the edge of the 50 ml. flask rather than its center, the absorption from intervening water should be comparable roughly to the absorption by tissues which screen the center of radiation of the gland.

SUMMARY

The construction and performance of a scintillation counter is described. The phosphor consists of a single crystal of NaI (T1) in a sealed moisture-proof container. The high efficiency of the counter for gamma radiation makes it suitable for a wide variety of clinical applications. For routine determination of thyroid uptake of I^{131} it may be placed far enough from the gland to eliminate serious errors of geometry. A one-sixteenth inch filter of lead is recommended to eliminate the appreciable augmentation of count due to back-scatter from tissue adjacent to the thyroid.

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THE ELECTROPHORESIS OF SERUM AND OTHER BODY FLUIDS IN FILTER PAPER

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IN 1937 Tiselius described the separation of the various fractions of protein in serum by electrophoresis, and since then many modifications have been described. All these suffer from the disadvantage that they are very expensive and complicated pieces of apparatus. When Cremer and Tiselius[1] showed that it was possible to obtain the same results with simpler apparatus, a method was introduced by which electrophoresis was brought within reach of the smaller laboratories. Modifications of the original apparatus have been described by Durrum,[4] Turba and Enenkel (1950), and Flynn and de Mayo[5] (1951), and various techniques by these authors and also by Knedel[7] (1951) and Griffiths[6] (1952).

The technique to be described has many advantages over those previously published, and can be carried out with relatively unskilled assistance.

APPARATUS

Rectifier.—A source of direct current is required. Fig. 1 shows the circuit employed in this laboratory, but any similar full-wave rectifier can be used.

Electrophoretic Tank.—This is made (Fig. 2) from 3/16 inch Perspex* sheet, and consists of a base containing seven small cells each measuring 7 by 6 by 5 cm. Each cell is separated from its neighbor by 2.5 cm. None of these dimensions are critical. Over the base fits a Lucite box, provided with a close-fitting lid, but no bottom as the support for the cells forms this. Six pieces of 1.5 cm. of Lucite rod are fixed transversly across the box so that they are positioned one between each of the cells and 18 cm. up from the bottom. In the center and two end cells is an electrode. This is made from 2 cm. of carbon rod. A piece of 2. B.A. screwed brass rod is soldered into a hole bored in one end of each electrode; on this is placed a 2.5 cm. rubber washer, and the rod is then passed through a hole in the side of the cell and screwed tightly, thus forming a water-tight joint. The two end cells are connected together and form the anode, while the center cell is the cathode. The pH change in the neighborhood of the electrodes is more marked at the anode than at the cathode, and by using two anodes and thus twice the amount of buffer this effect is minimized. When the cells are bridged by paper strips it will be seen that there are two lots of three cells connected in series and that these are then connected in parallel.

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*Perspex is a transparent, plastic acrylic sheet, and is the trade name of Imperial Chemical Industries Ltd., 2, Buckingham Gate, London,S. W. 1, England. Similar substances are marketed in the United States under trade names Chrystalite, Lucite, and Plexiglas.

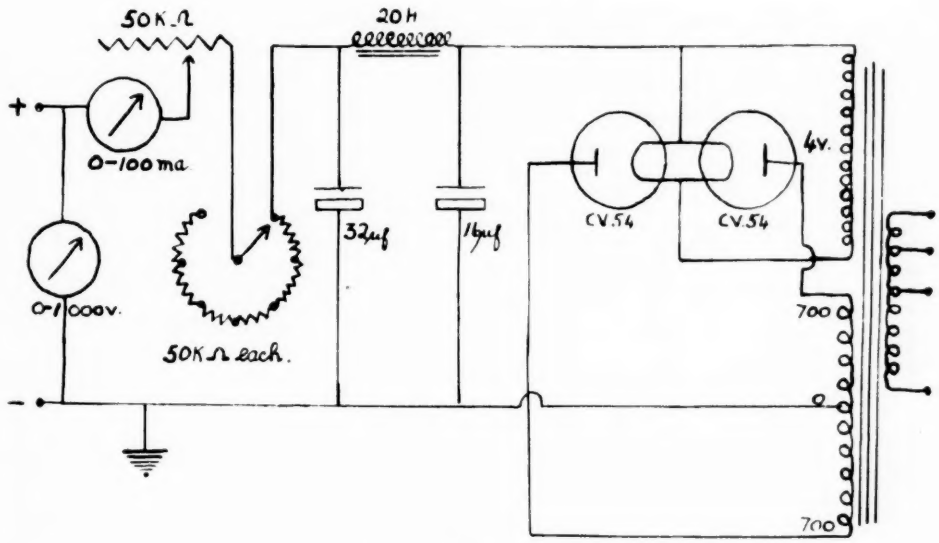
50K Ω
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Fig. 1.

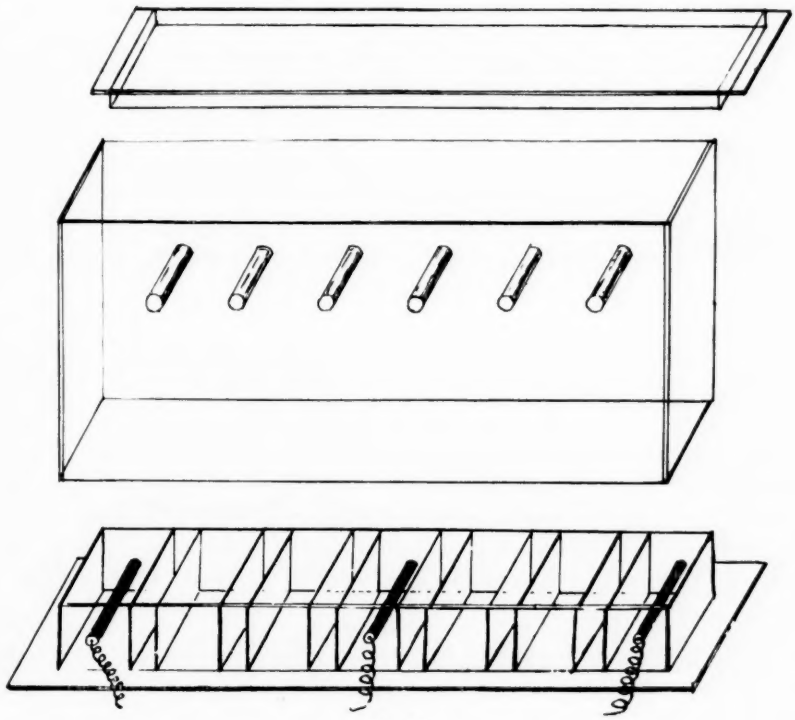

Fig. 2.

TECHNIQUE

Reagents Required.—

1. Strips of Whatman No. 3 filter paper, 36 by 5 cm., with a pencil line drawn across the center.
2. Barbitone Buffer, pH 8.6 Durrum (1950). 0.05M diethylbarbiturate and 0.01M diethylbarbiturate acid. The tank holds one liter.
3. Dye. 1 per cent bromphenol blue in ethanol saturated with mercuric chloride.
4. Dioxan, commercial grade.*
5. Pieces of glass to fit sink and several spring paper clips 3 inches long and 1 inch wide.

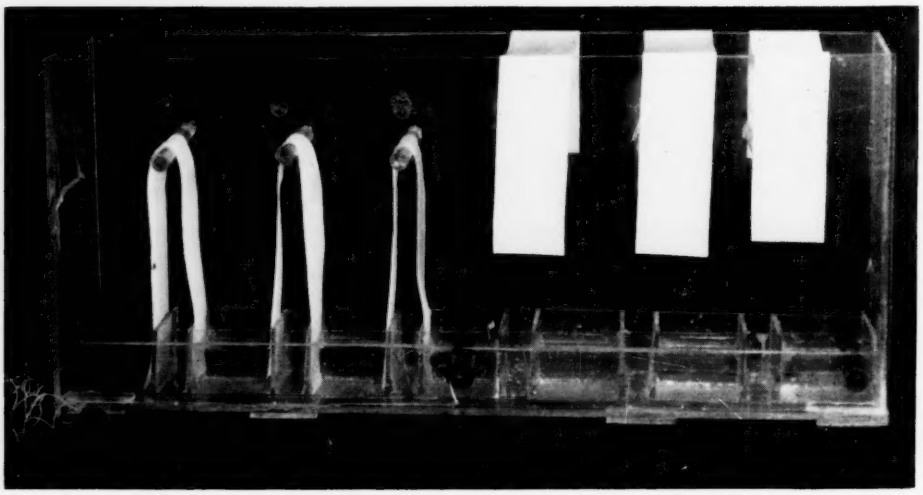

Fig. 3.

Procedure.—The cells are filled with buffer to within 1 cm. of the top. Six strips of filter paper are taken, soaked in buffer, and blotted with a clean towel to remove excess fluid and placed across the top of the tank. Owing to the softness of the wet paper, the strip becomes slightly concave. It is then easy to apply 0.1 ml. of serum or urine to the pencil line, the concavity preventing the applied fluid from spreading too far. When the serum has been absorbed the strips are placed over the Perspex rods with the ends dipping into alternate cells. The ends of the strips are tapped with a spatula into contact with the cell wall to which they adhere by capillary attraction. The lid is replaced and the electrodes connected to the rectifier and the current switched on. It is advisable to make a rule that the interior of the tank must not be touched unless completely disconnected, as this voltage can be lethal; even when switched off the condensers hold a charge suffi-

*The remarkable results of Dioxan are due to an unknown impurity. The best results are given by Judex brand made by the General Chemical and Pharmaceutical Co., Sudbury, England. This Dioxan is of U. S. A. origin.

cient to give a shock for some considerable time. Fig. 3 shows the tank set up with three papers in position and three more in the position for inoculating the serum.

A constant current is mantained for six hours. The strip is then dried overnight at 70° C. or 105° C. for thirty minutes and immersed in the dye for exactly five minutes. It is then clipped to the glass plate and washed in running water until the background is almost white (about twenty minutes) and immersed in Dioxan for five minutes. The Dioxan is poured off and replaced by ether for five minutes; and the strip is dried in a current of warm air. It dries almost immediately and is then ready for scanning.

DISCUSSION

Apparatus.—The electrophoretic tank of Flynn and de Mayo[5] is extremely satisfactory. It, however, has been designed to run at a low current for twenty hours. Modification by the introduction of thicker electrodes effects an improvement, and the tank can be used at 3 Ma. per strip for seven hours with very good results. As the strips are in parallel unless they are of exactly equal resistance they will carry different currents, and we have had irregular migration from this cause. In order to be quite sure that the current is always 5 Ma. in each strip, we prefer the strips to be, as far as possible, in series. It is thus possible to produce perfect results in six hours, and as the fractionation takes place during working hours it is possible to ensure that the current does not vary. The Flynn and de Mayo tank works well at the high current with up to four strips, but with six strips at least 10 watts have to be dissipated, and resistances of this order are bulky and expensive. By having the strips in series the paper itself takes part of the load. An even voltage drop taking place between the ends of the strip and resistances in the unit can be reduced to 5 watts with safety. If all the cells are connected in series a very high voltage would be required as each strip has an initial resistance of 60K ohms. The total in series would be 360K ohms, while the series parallel connection reduces this to the more workable one of 90K ohms. The total current will be 10 Ma. and will therefore require a voltage of 900. Although the transformer is rated at 700 volts, it will be found that the small drain of 10 Ma. has very little effect on the no-load voltage, and consequently the rectifier will be found to produce over 1,000 volts.

Rectifier.—This follows the usual full-wave practice and uses a 700-0-700 volt transformer with two C.V. 54 high voltage valves in parallel. Smoothing is by choke and condenser, and the current passes through a series of 5 watt 50K resistors. A rotary stud switch is used, as the high wattage will burn out

TABLE I

	WEIGHED	PLANIMETER	TRAPEZOID	SIMPSON	TECHNICIAN
Albumin	31.2	31.2	30.6	30.5	30.8
$\alpha 1$	10.8	10.5	10.3	10.6	10.8
$\alpha 2$	15.9	16.0	16.5	16.9	16.2
β	18.7	18.3	18.1	18.3	18.0
γ	23.4	24.0	24.6	23.7	24.2

the contacts of the ordinary wafer type switch used in wireless sets. This forms the coarse control, the fine control being a 10 watt 50K potentiometer. All the necessary parts for the rectifier will be found in the surplus "Power Rectifier" No. 392. This cannot be used as purchased, but must be rewired and the voltage limiting 10 watt 5K resistor removed.

It will be found that the initial strip resistance of 60K has at the end of one hour dropped to 30K, and consequently the current will rise. During this period a watchful eye must be kept on the milliammeter, and the current reduced to 10 Ma. when necessary. At the end of the hour conditions become stabilized, and only an occassional inspection is necessary. During the run all the proteins will migrate toward the anode, but due to endosmosis, the heavier gamma globulins will be found to have actually moved back toward the cathode. This migration occurs at a constant rate. Fig. 4 shows the result of running strips for different periods, the current being constant. The distance migrated, if plotted, will fall on a straight line. At the end of two hours β globulin has commenced to separate, at four hours γ, β and α_2 can be seen while, although α_1 commences to separate at five hours, it is not completely separated until six hours. At the end of seven hours separation is complete and any further period of run only results in blurring the pattern.

In spite of considerable evaporation and deposition on the inside of the tank, it will be found that the temperature of the strips never rises more than two degrees above air temperature. Until experience has been gained, it is useful to employ a visable marker. This can consist of a jaundiced serum, the bilirubin moving with the albumin fraction, or, more conveniently, a normal serum to which has been added a small knife point of dry bromphenol blue. In this case also the dye migrates with the albumin fraction and it forms a vivid and easily seen marker.

Paper.—In the early days of testing out a successful technique we tested a great number of different papers. It soon became clear that certain attributes were required in a suitable paper. These were maximum absorption, absence of fluffiness, and a hard tough surface. Several fairly satisfactory papers were found, the best being Whatman drop reaction, and 3 mm. of Whatman No. 3 was found to be ideal. It is capable of absorbing up to 0.5 ml. of fluid and is so tough that it can be washed vigorously under a tap.

Buffer.—We find that a barbiturate buffer of pH 8.6 and ionic strength 0.1 to be most satisfactory (Durrum, 1950). The citrate barbiturate buffer of Deutsch and Goodloe[3] (1945) has not in our hands yielded satisfactory results. Experiments using buffers of higher and lower pH values and different ionic strengths have uniformly given less separation.

Dye.—Until recently a satisfactory dye had to be capable of being completely eluted from the paper. This no longer holds as we (Griffiths,[6] 1952) have shown that satisfactory results can be obtained by direct reading of the paper strip. We have therefore examined a number of dyes, all of which were of 1 per cent concentration dissolved in ethanol saturated with mercuric chloride. In a few cases where the solubility of the dye was less than 1 per cent, a

saturated solution was used. All strips were washed in water except where this failed to produce a clear background. The following dyes were found to be useless, either because they failed to be absorbed onto the protein, or because it was impossible to wash them out of the paper: brilliant cresol blue, thymolphthalein, toluidine blue, aniline blue, Alizarin, Congo red, crystal violet, vital carmine, indigo carmine, indulin, vital isamine blue, methyl violet, Jodgrun, Janus green, Malachite green, methylene green, magenta, trypan blue tartrazine, orange 111, soluble blue, neutral red, nile blue, Scharlach R, carmine, Bismarck brown, Safranin, methylene blue, rhodamine, and azorubin.

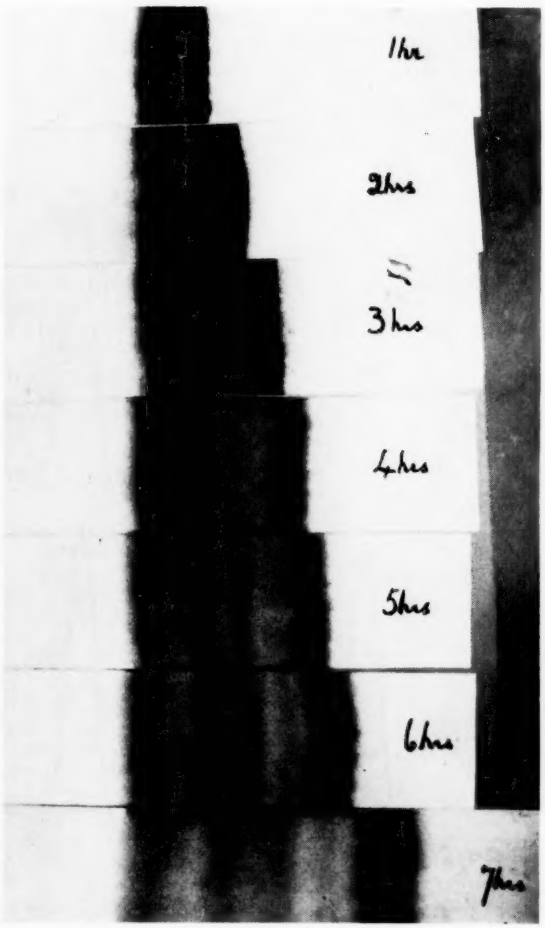

Fig. 4.

Saüre violet and Biebrich scarlet give very good strips, but are very difficult to wash out of the paper, and the final washes have to be with ethanol. Bromeresol blue and bromthymol blue give good results, much better than either azocarmine or naphthalein black which are extensively used. Light green and orange G also give satisfactory strips but the color is weak; both

these dyes can be washed out of the paper very easily and will be worth investigation to discover a more satisfactory technique.

For general use we prefer to use bromphenol blue in the technique described. By washing entirely with water the tedious and expensive washing with methanol as recommended by Flynn and de Mayo is avoided. It is possible to wash the strip until the background is white, but if this technique is adopted it will be found that in those strips which contain a large proportion of albumin the dye will diffuse during the drying period and the background will no longer be perfectly white. This will later cause trouble when attempting to set the galvanometer to zero. For this reason we prefer to wash until the background is almost white, and immerse the strip in Dioxan. Dioxan has the property of bleaching unadsorbed dye. The dye is soluble in Dioxan, but is unionized, and a strip with a clear white background results. We recommend ether to remove the Dioxan and hasten drying.

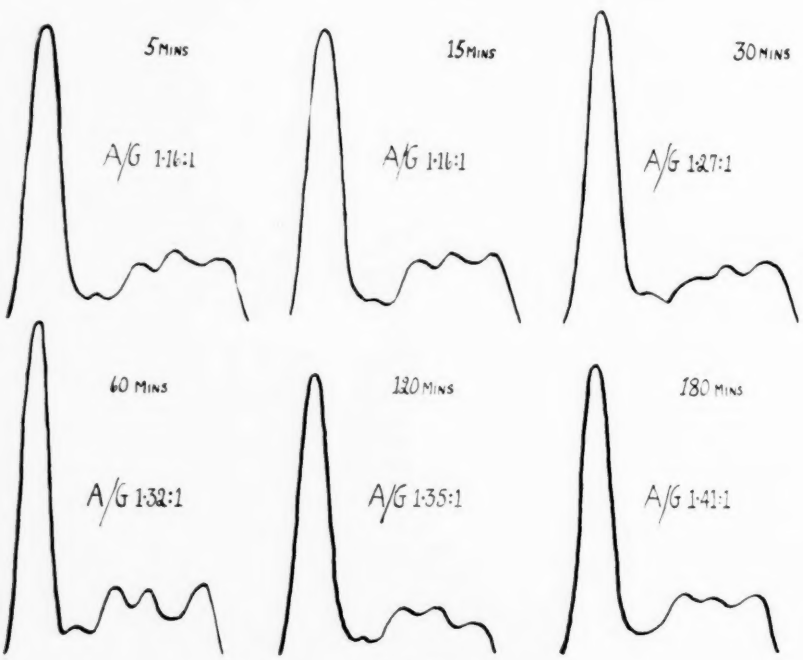

Fig. 5.

We can find no foundation for the statement that the blue dye is more strongly absorbed by the albumin fraction and that the globulin fractions have to be multiplied by the arbitrary factor of 1.6 (Cremer and Tiselius, 1950). We have confirmed the finding of Flynn and de Mayo that prolonging the staining time will alter the ratio of the fractions as albumin will absorb more of the stain than globulin, but by our technique this effect is much less marked. Fig. 5 shows the results from six strips run under the same conditions but stained for different periods of time. It will be seen that with gross overstaining the

albumin/globulin ratio gradually increases but that up to five times the recommended time of staining does not alter the pattern.

Reading.—The dyed strip can be cut into sections and the dye eluted and the resulting blue solution read in a photoelectric colorimeter. This is a laborious method and requires on the average 20 to 25 readings a strip. For this reason we prefer to read the strip directly (Griffiths, 1952). It has been stated by Cook, Harris, and Warren[2] (1952) that the transmission readings obtained in this way are not logarithmic but hyperbolic. They, however, used azorcarmine and con-

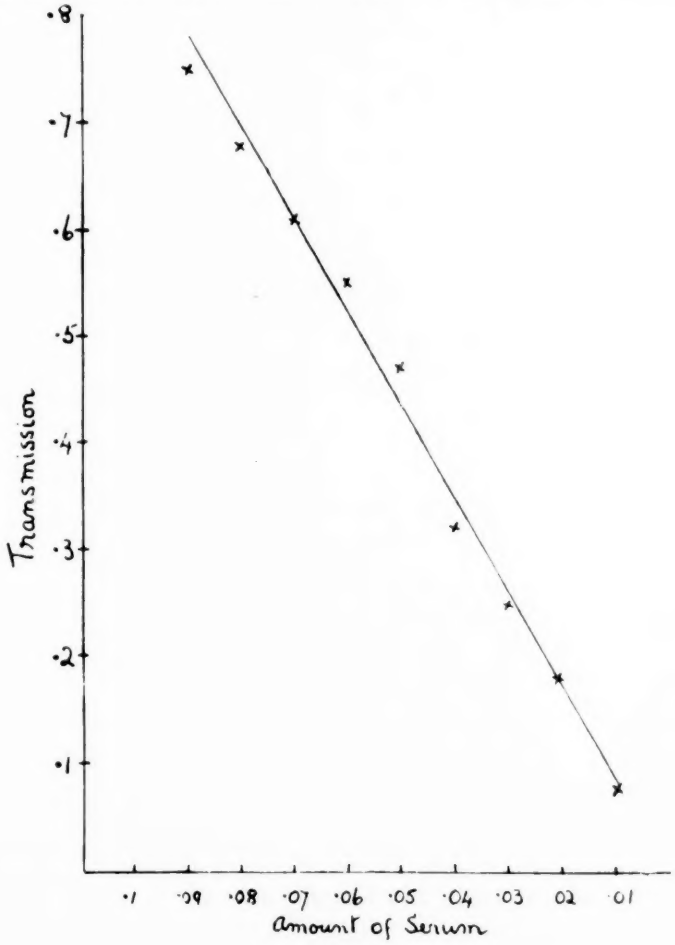

Fig. 6.

structed their color wedge by soaking pieces of filter paper in different dilutions of serum. We have found it impossible to wash two pieces of paper so that the total color in each is identical. The relative proportion in one strip compared with another is identical, but the total color even in two strips treated side by side under the same conditions varies. Fig. 6 shows the results of an experiment in which serum was diluted so that known amounts were contained in a constant

volume of 0.1 ml. These were applied to a series of pencil lines on one long strip in the usual way and dried and stained. The strip was then read in the scanner and the total transmission of each colored area, usually six or seven readings, was plotted against the amount of serum. It will be seen from the graph that a straight line results and that there is substantial agreement with Beer's law.

Calculation of Results.—We prefer to use 1 inch squared paper as it gives a larger graph, and for this reason our scanner has a 1/10 inch slit. This results in the length of the graph being the same as the length of the colored area

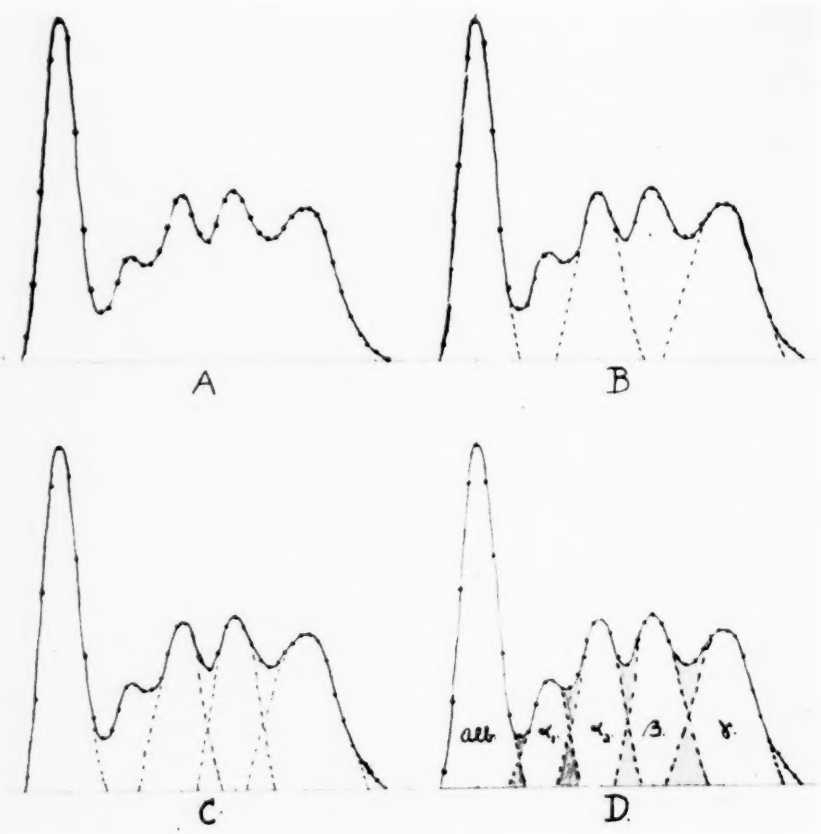

Fig. 7.

on the strip, and is an aid in finding the base line in those cases where the graph does not come to zero. The plotted curve is analyzed by the method of Petersen (1945).[8] Fig. 7 shows the successive stages in the construction of a graph. *A* shows the points joined by a continuous line; *B* shows the filling in of the more obvious peaks by regular curves; *C* is a further stage; and *D* is the complete graph. When complete, the area under each curve is measured, and the percentage of the total area is calculated. It may be felt at this stage that these curves are arbitrary and that it is possible to draw other curves which will give

different results. Table I column 5 shows the result of giving the readings from which curve *A* of Fig. 7 was plotted to an experienced technician who had no knowledge of the figures already calculated. Her curve when superimposed on Fig. 7, *D* was almost identical, and the actual figures obtained are shown in the first and last columns and can be compared.

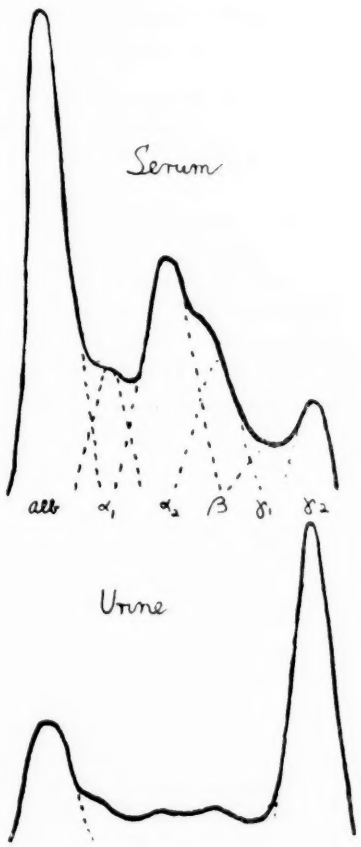

Fig. 8.

The estimation of the area can be carried out accurately by using a planimeter, a relatively expensive instrument and one which requires a great deal of experience before consistent results can be obtained. If the graph is drawn on a good grade paper the curves can be cut out and weighed, the weight being proportional to the area. Alternatively the area can be calculated by using the trapezoid rule or the more accurate Simpson's rule. Proof of these two rules can be found in any elementary book on calculus. Fig. 8 shows the results obtained on curve *D* of Fig. 7 using these four methods. Any laboratory possessing an aperiodic balance will find weighing much less trouble and equally accurate. The percentage figures thus obtained are applied to the figure for the total protein, obtained by any standard method and the absolute figures reported.

This method of analysis has one great advantage. In cases where the urine contains protein, both serum and urine can be run at the same time. Fig. 8 shows serum and urine from a patient with multiple myeloma. This shows an inverse ratio between the concentration of albumin and gamma globulin in the patient's serum and urine. This urine gave an atypical Bence-Jones reaction.

My thanks are due to my Senior Biochemical Technician, Miss V. Brews for her help in this investigation, and to Mr. E. Poynter, the Group photographer, for the photographs.

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THE CHOLESTEROL CONTENT AND IODINE NUMBER OF HUMAN SEBUM

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ONLY in recent years has sebum been subjected to exact chemical analysis. The most complete study is that of Mackenna and associates[1] whose data indicate that sebum is composed of equal parts of free fatty acids (2/3 being unsaturated), combined fatty acids, and an unsaponifiable fraction. Of the latter, 14 per cent was found to be cholesterol, 46 per cent hydrocarbons, and 8 per cent saturated and unsaturated alcohols.

Other than the work of Kirk,[2] concerned predominantly with skin lipids of elderly subjects, and that of Eckstein,[3] concerned with the lipids of human hair, correlations of chemical variation in the sebum with age and sex have not been recorded to our knowledge. This paper deals with variation found in cholesterol content and iodine number of sebum as affected by sex, puberty, menstrual cycle, and certain disease states, especially acne vulgaris.

Acne was of particular interest because it seemed possible to us that sebaceous gland plugging, an essential event in the pathogenesis of acne vulgaris, might be explained by an increase in the saturation of the lipids contained therein and a concomitant increase in melting point.

METHODS

Collection of Skin Surface Lipids.—The collection of surface lipids was accomplished by the method of Ricketts and associates,[4] as modified and described by Mackenna, Wheatley, and Wormall.[1] The essential points, as we employed them, are as follows: each forearm and hand were immersed in distilled acetone for a period of three minutes. The acetone was evaporated at 100° C. leaving a residue of fat, dirt, cellular debris, a small amount of water, and acetone. The remaining acetone and water were removed under reduced pressure at 100° C. The residue was dissolved in 10 to 15 ml. of chloroform, filtered through Whatman No. 2 paper into a previously weighed 50 ml. flask. The chloroform and any remaining acetone or water were carefully removed at reduced pressure in a warm water bath. The sample became a homogeneous molasses-like substance, which did not change in weight or appearance with further gentle heating and evaporation over a thirty- to sixty-minute period. To test the reproducibility of the weight of the residue several samples were redissolved in chloroform and the evaporation repeated. The observed weights agreed within 0.8 per cent. The weight of the extracted sebum was found to average 50 mg. in children and 130 mg. in adults. The sample was dissolved in a known quantity of chloroform so that 3 to 5 mg. of sample was contained in each milliliter. Samples were kept in tightly stoppered 50 ml. flasks, and were refrigerated until analyses could be performed.

Most subjects were warned in advance to avoid contamination of the skin, and all were carefully questioned prior to extraction about the recent use of hand lotions and the

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possibility of extraneous fatty substances (butter, grease, etc.) on their hands and forearms. When these substances were thought to be present, extraction was deferred.

Acetone was used since both Ricketts and associates[4] and Mackenna and associates[1] were impressed by the lack of untoward reactions in their subjects. Except for transitory post-extraction dryness, we had only one instance of mild irritation and erythema of the forearm in our series of 71 extractions from 57 individuals.

Sebum specimens were obtained from normal healthy volunteers and patients of Barnes Hospital, the St. Louis Children's Hospital, and Washington University Clinics.

Collection of Sebaceous Plugs.—Sebaceous plugs were collected with a comedone extractor from the face and upper trunk of patients with acne vulgaris. They were refrigerated until a sufficient quantity had been obtained for the determination of iodine and cholesterol values. The comedones were crushed, placed in a weighed flask, and dried over calcium chloride for twenty-four hours. The dried plugs weighed 23 mg. and 157 mg. in our two samples. About 400 ml. of acetone was added to the plugs which were then allowed to stand for two hours. From this point they were treated in a manner exactly similar to the forearm samples.

Iodine Number.—The determination of the iodine number was done by the method of Yasuda[5] using 3 to 5 mg. of lipid. Each sample was run in triplicate.

Intermittently, to check the accuracy of the method, determinations were run on pure cholesterol (theoretical iodine value of 65.8) dissolved in chloroform. Results ranged from 65.9-71.2, i.e., within 8 per cent of the theoretical iodine value.

Analyses repeated at a later date on three sebum specimens demonstrated reproducibility within 2 to 3 per cent.

Cholesterol.—The method of Kingsley and Schaffert[6] has been adapted for the measurement of sebum cholesterol. The actual technique used was as follows:

A 1 ml. aliquot of chloroform solution of skin surface lipid (3 to 5 mg.) was added to 9 ml. of chloroform in a stoppered glass tube. In the same manner, 2 ml. of cholesterol standard (0.1 mg. per milliliter) was added to 8 ml. of chloroform. The solutions were cooled in ice water for fifteen to thirty minutes, and 1.5 Gm. of U.S.P. anhydrous magnesium sulfate was added to each. All tubes were shaken rapidly by hand for five minutes, cooled again to keep evaporation at a minimum, and filtered through Whatman No. 2 paper (a process which took about two to four minutes). A 5 ml. aliquot was then transferred to a spectrophotometer cuvette, corked as before, and again cooled. Thereupon 2 ml. of cold, freshly prepared 4:1 acetic anhydride-sulfuric acid reagent was added; the solution was mixed, and immediately read in the Coleman No. 11 spectrophotometer at 625 mu against a chloroform blank. Readings were taken every two to three minutes until a minimum transmittance was obtained. All determinations were run in duplicate.

We found by means of a standard curve that Beer's Law applied to concentrations of 0 to 0.5 mg. cholesterol per 5 ml. chloroform under the above conditions. A cholesterol standard was run with every series of unknown. Cholesterol concentrations were calculated from the optical density of the cholesterol standard.

Accuracy of the method was tested by the addition of a known amount of cholesterol roughly equal to that amount present in each of eleven sebum samples. The mean recovery was 99 per cent, and most values ranged between 90-110 per cent.

The reproducibility of the technique was indicated by the values obtained on six samples repeated on different days. The maximum difference noted on subsequent analysis was 1.4 per cent cholesterol.

"Cholesterol" is used in this paper to mean any sterol which produces a color absorbing at 625 mu. There is the possibility that 7-dehydrocholesterol[7] and perhaps other skin lipids will react to form color under the conditions used.

Cholesterol ester produces a slightly greater color density than does the same concentration of free cholesterol molecules by this method. An empirically derived correction factor may be used to obtain a truer estimate of total cholesterol in serum.[6] However, in this study the cholesterol values are uncorrected. Therefore, our "cholesterol" concentrations are slightly elevated proportional to the amount of ester contained in the sample.

RESULTS

Cholesterol Values of Skin Surface Lipids.—The mean cholesterol concentration of the skin surface lipids extracted from the forearms of adults was found to be 5.2 per cent by weight, and did not differ significantly between males and females of the same age range (Table III). Ricketts and associates[4] and Mackenna and associates,[1] whose collection method we have used, obtained mean values for adults ranging from 4.4 to 6.75 per cent. Cholesterol concentrations reported by Kirk,[2] who collected sebum from the forehead, were 2.0 per cent for men, aged 16 to 39 years, and 2.2 per cent for females of the same age group. Kyle,[8] who obtained sebum by swabbing the face and upper trunk, reported a cholesterol value of 2.3 per cent in normal subjects of the acne age range.

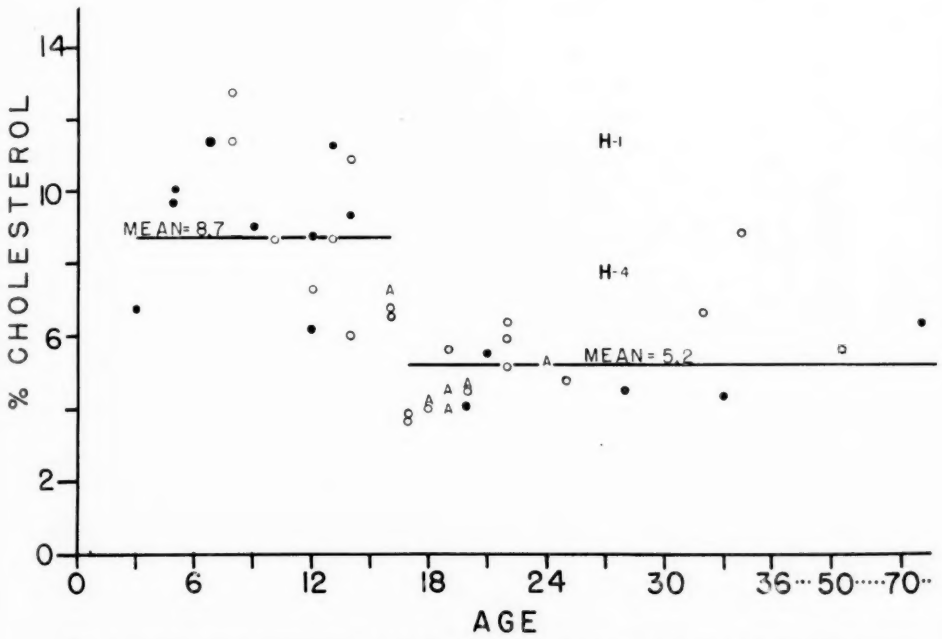

Fig. 1.—Cholesterol concentration of sebum is plotted against age of subject. Males represented by solid dot, females by circles, acne subjects by A. *H-1* represents hypogonad individual after one week of 15 mg. buccal testosterone per day, *H-4* is same individual after four weeks. All samples except *H* were used in calculation of the means.

A significant decrease in sebum cholesterol value was found to occur at puberty (Table III and Fig. 1), a fact quite consistent with Eckstein's[3] finding of 10.1 per cent cholesterol in lipid from children's hair and 2.6 per cent in the hair of adults. We suggest that this change is related to the sex hormones. Additional evidence for this hypothesis is that the sebum cholesterol collected after one week of testosterone therapy from a 27-year-old male patient who showed evidence of testicular insufficiency and growth failure fell into the range found to be normal for prepubertal subjects. However, after three additional

weeks of therapy the value had fallen from 11.5 per cent to 7.6 per cent. On the other hand, we found no significant correlation of cholesterol concentration with the day of the menstrual cycle (Fig. 2, *A*).

Although hormonal changes are probably in some way concerned in the etiology of acne vulgaris, we found no significant differences in cholesterol content of sebum from the forearm of normal subjects and of persons with acne in the same age range (Table III). Furthermore, our one determination of

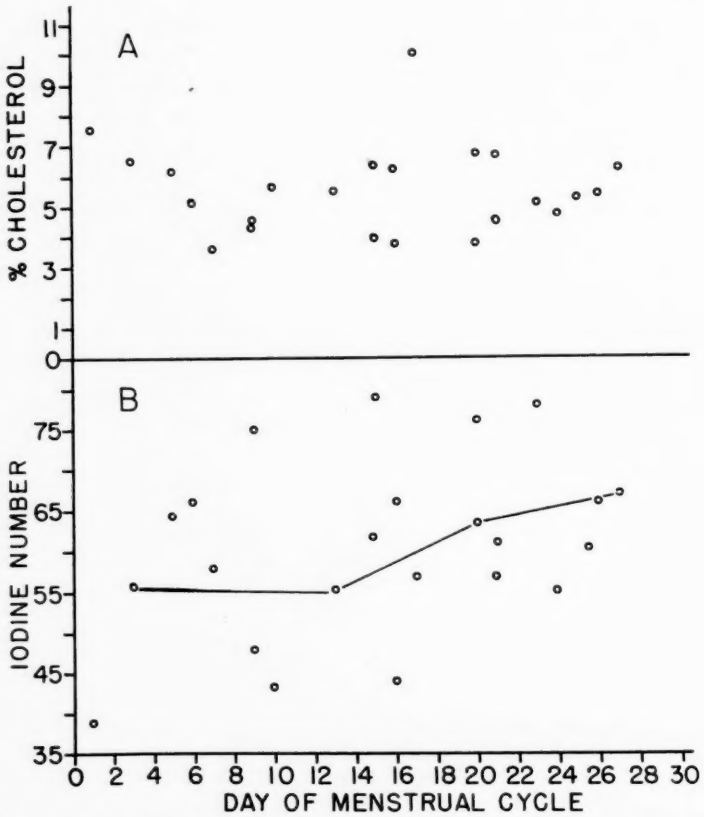

Fig. 2.—*A*, Sebum cholesterol concentration plotted against day of menstrual cycle demonstrates no correlation. These samples were obtained from 14 females. *B*, Iodine number is plotted against day of menstrual cycle. Calculated slope of 0.176 was found to be not significant ($P > 0.1$). Samples were obtained from 14 females. Results from 4 samples obtained from same female at weekly intervals are connected by the solid lines.

cholesterol content of lipid derived from sebaceous plugs (6.9 per cent) did not vary grossly from that of normal skin secretions. These results corroborate Kyle's[8] findings of similar cholesterol concentrations in normal and acne surface lipids as well as in comedones.

To determine whether a relationship exists between serum cholesterol levels and concentrations of sebum cholesterol, the cases studied are shown in Table I

These fragmentary data do not suggest any such correlation.

TABLE I

NAME	DIAGNOSIS	AGE	SERUM CHOLESTEROL	% CHOLESTEROL IN SKIN SECRETION
H. M.	Chronic Glomerular Nephritis	3	360 Mg. %	6.8
W. B.	Chronic Glomerular Nephritis	7	(Serum lipemic)	11.3
M. J.	Thyrotoxicosis	14	112 mg. %	10.8

TABLE II. CHOLESTEROL CONCENTRATION AND IODINE NUMBER OF SEBUM

	CHOLESTEROL CONCENTRATION				IODINE NUMBER			
	MALES		FEMALES		MALES		FEMALES	
	AGE	CHOLESTEROL	AGE	CHOLESTEROL				
	YEARS	PER CENT	YEARS	PER CENT	AGE	I#	AGE	I#
Normal	5	9.6	8	12.7	5	44	8	65
	9	9.0	8	11.4	9	51	8	47
	12	6.1	10	8.6	12	40	10	47
	12	8.7	12	7.2	12	44	12	65
	13	10.6	13	8.7	13	71	13	51
	14	9.4	14	6.0	14	68	14	52
	20	4.1	16	6.3	20	80	16	78
	23	5.6	16	6.2	23	66	16	66
	28	5.5	17	3.9	28	60	17	62
	33	4.2	17	3.7	33	70	17	44
	78	6.3	18	4.0	78	76	18	60
			19	5.6			19	41
			20	4.6			20	63
			22	5.8			22	65
			22	5.1			22	63
			22	6.2			22	60
			25	4.7			25	55
			32	6.6			32	57
			34	8.8			34	56
			55	5.7			55	55
Acne	16	7.2	19	4.5	16	54	19	79
	18	4.1	19	4.0	18	73	19	76
	20	4.7			20	64		
	24	5.3			24	75		
Diagnosis								
Chronic glomerular nephritis	3	6.8		3	55			
Nephrotic stage of chronic glomerular nephritis	5	10.0		5	38			
Chronic nephritis	7	11.3		7	52			
Thyrotoxicosis			14	10.8			14	66
Hypogonad (1 wk. testosterone Rx)	27	11.3		27	65			
Hypogonad (4 wk. testosterone Rx)	27	7.6						
Anhydrosis	28	6.2		28	98			
Anhydrosis (after ACTH)*	28	5.5		28	89			
Comedone batch #1				16-28	96.4			
Comedone batch #2 (Males and females)	21-27	6.9		21-27	76			
Negro	9	7.3		9	49			

*80 mg. parenterally over a two-day period.

TABLE III. ANALYSIS OF CHOLESTEROL DETERMINATIONS*

	COMPARISON OF		COMPARISON OF		COMPARISON OF		COMPARISON OF	
	CHILD FEMALES	CHILD MALES	ADULT FEMALES	ADULT MALES	NORMAL MALES AND FEMALES	ACNE MALES AND FEMALES	CHILDREN ALL MALES AND FEMALES	ADULTS ALL MALES AND FEMALES
Age in years	8-16	3-16	17-54	17-80	16-24	16-24	3-16	17-80
Number of samples	10	11	23	8	19	7	21	31
Number of individuals	9	10	14	7	12	6	19	21
Mean % cholesterol for individuals	8.65	8.87	5.31	5.10	5.09	4.97	8.71	5.24
Standard deviation	2.45	1.69	1.62	0.801	0.99	1.19	2.03	1.39
Standard error of mean	0.82	0.54	0.434	0.317	0.285	0.486	0.466	0.303
F test† for ratio of variance	$P > 0.1$		$P < 0.05$		Not necessary because variances nearly equal		$P < 0.05$	
Probability	$P > 0.5$		$P > 0.5$		$P > 0.5$		$P < 0.001$	

*Includes all individual determinations listed in Table II except those from hypogonad, the anhydrotic and the Negro subjects.

†When variances were so unequal that P was less 0.05, we followed the currently correct practice of deriving the t ratio and Degrees of Freedom (for the t test) by means of the formulas given in Dixon and Massey: Introduction to Statistical Analysis. McGraw and Hill, 1951, p. 105. Otherwise t was determined as is usual from the ratio D/sD with D.F. $= n_1 + n_2 - 2$.

Iodine Numbers of Skin Surface Lipids.—The results of the determination of the iodine number of sebum demonstrated an increase of iodine number with age among male subjects (Fig. 3). A regression line with a slope of 0.996 was found to be statistically valid. No comparable rise in fatty acid unsaturation with age was found in sebum from females (Table II). Sebum of subjects with acne did not show any definite alteration of iodine number as shown in Table IV. The sebum from sebaceous plugs was found to be equal to or somewhat more unsaturated than that from the forearm (Table II).

Consistent alteration of iodine number with the menstrual cycle did not occur (Fig. 2, *B*).

The individual with anhydrosis had the highest iodine number in our study (Fig. 3), suggesting that the sebaceous gland secretes sebum with a high iodine number. In the normal person this high iodine number may be lowered by dilution with lipids from the sweat glands.

TABLE IV

	NORMAL (10 FEMALES) (2 MALES)	ACNE (2 FEMALES) (4 MALES)
Age Range	16-24	16-24
Number of individuals	12	6
Iodine number mean	62.25	70.16
Standard deviation	11.14	9.4
Standard error of mean	3.22	3.85
F test for ratio of variance	$P > 0.1$	
t test for significance between means	$P > 0.1$	

The samples of the following subjects did not differ from normal white subjects of the same age range (Table II): (a) three males with nephritis, (b) one female child with thyrotoxicosis, (c) one male aged 27 years who showed evidence of testicular insufficiency and growth failure, and (d) one normal Negro male child.

DISCUSSION

Sebaceous glands are known to increase in size and number at the time of puberty. Our studies demonstrate a pubertal change in the composition of sebum in that there is a sharp drop in cholesterol concentration.

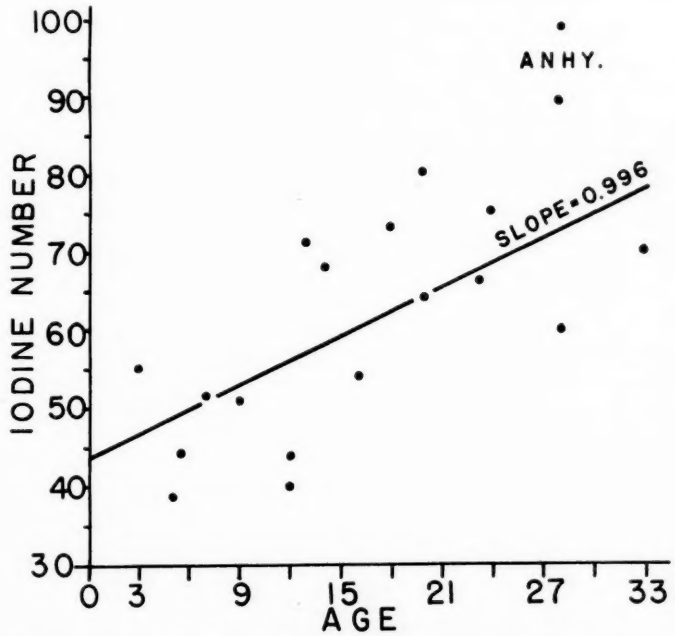

Fig. 3.—Iodine number of sebum is plotted against age of male subject. Statistically there is less than one chance in 100 that such a relationship is due to chance alone. Dots bounding ANHY represent samples from anhydrotic patient on two different occasions.

It is logical to suppose that the sex hormones are related to these pubertal changes in the sebum. This view is supported by our incomplete observations on a male hypogonad during methyl testosterone therapy. Because the drop in cholesterol concentration occurs in girls as well as boys, the alterations in sebum may be provoked by estrogens as well as androgens. Our failure to find a correlation of sebum constituents with the menstrual cycle suggests that the sebum is not altered by rapid changes of estrogen level. The relative efficiency of androgens and estrogens in producing the adult type of sebum can be evaluated only by much more extended studies of sebum from hypogonadal patients receiving hormonal replacement therapy or of children with adrenal or ovarian lesions associated with excessive sex hormone production.

The significance of the high cholesterol content of sebum from children is not apparent. It is tempting to speculate teleologically that the high cholesterol content might mirror a correspondingly high concentration of vitamin D precursors in the skin. This would be of advantage to the child during the period of rapid growth.

Our studies, like those of Kyle,[8] have failed to demonstrate any chemical or physical alteration of sebum of sebaceous plugs which might be significantly related to the pathogenesis of acne. Furthermore, our incidental corroboration of Schmidt-Nielsen's[9] finding that lipid comprises only approximately 25 per cent of the dry weight of the sebaceous plugs leads us to de-emphasize the role of lipid changes in the pathogenesis of sebaceous gland plugging.

SUMMARY

Cholesterol and iodine number determinations have been carried out on 60 samples from 45 individuals.

A significant postpubertal drop in sebum cholesterol concentration in male and female subjects has been demonstrated. A sample from a 27-year-old man showing evidence of testicular insufficiency and growth failure had a value similar to those in the prepubertal range, and this decreased during testosterone therapy. There was no significant difference in sebum cholesterol between males and females of similar age.

A significant increase in the sebum iodine number with age has been shown in male subjects. The increase did not occur in females.

No correlation of cholesterol concentration or iodine number with the menstrual cycle was found.

Sebum from a patient with anhydrosis showed the highest iodine number of our series.

It is suggested that the amount of cholesterol present in the skin surface lipids may be influenced by sex hormone levels and may reflect the concentration of vitamin D precursor in the skin.

A hypothesis concerned with lipid saturation in the etiology of sebaceous gland plugging was not supported by our data.

We wish to express special appreciation to Dr. William Daughaday and the staff of the Metabolism Laboratory of Barnes Hospital for encouragement and cooperation in the carrying out of this project.

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THE EFFECT IN MAN OF A NEW HEPARINOID, TREBURON*

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THE search for new anticoagulants with heparinlike activity has recently resulted in the development of an active polyhexuronic acid ester derived from pectic acid to which the name of Treburon has been given. The basic pharmacologic response in animals has been reported by Mangieri and coworkers[1] and Hirschboeck and associates[2] have already recorded initial clinical experiences with Treburon. A study of the effects of the drug in man has been under way in these clinics for several months. In the present report are recorded the control studies in which Treburon and heparin are compared, while the therapeutic experiences will be reported separately.†

EXPERIMENTAL

For determining clotting times a modified Lee and White technique[4] was employed. Equipment consisted of scrupulously clean 12 by 40 mm. test tubes, No. 00 rubber stoppers, 18-gauge needles, 10 ml. syringe, and a constant temperature water bath. After smooth venipuncture 3 to 10 ml. of blood was collected, the syringe detached, and discarded. A fresh dry syringe was attached to the inserted needles, and 5 ml. of free-flowing blood was rapidly obtained with absence of stasis or bubbling. A stopwatch was started, one ml. blood was placed in each of 3 tubes, and the rubber-capped tubes were placed in the water bath at 37 ± 1° C. After five minutes the first tube was completely inverted, and this procedure was repeated at intervals of one minute, until a firm clot had formed. The second tube was tilted at one-minute intervals until a clot had formed, and then the third tube. The clotting time was taken as the time ensuing from drawing the blood until the last firm clot had formed. Specimens clotting in five minutes or less were repeated to eliminate the possibility of thromboplastic contamination of the sample. Samples taken from the same patient over several days were remarkably reproducible under these conditions, and the average clotting time of 500 samples taken from 200 patients with a variety of minor disorders was 13 ± 6 minutes. It is generally believed that the clotting time has to be prolonged at least twice that of normal to demonstrate therapeutic effect. Prothrombin times were done by a diluted plasma technique.[5] About 200 subjects were used in these studies from the wards of the Los Angeles County General Hospital; they were unselected as to age and sex. In the examination of the effect of Treburon and heparin in control human subjects it was necessary to omit those who were acutely ill and those suffering from bleeding dyscrasias. A separate study of the drug was made in patients with renal and hepatic diseases. The heparin was supplied‡ as the heparin sodium salt, and the Treburon also was administered in a simple isotonic solution with preservative, no repository menstruum being used.

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*Treburon is the trademark of the sodium salt of sulfated polygalacturonic acid methyl ester methyl glycoside developed in the laboratories of Hoffmann-La Roche, Inc., Nutley, N. J., and furnished by Dr. Leo A. Pirk.

†Transient but severe alopecia as a curious and unexpected delayed effect as well as diarrhea obtained with even relatively small doses will in all probability preclude the clinical use of Treburon, and this will be described in a subsequent paper.[3]

‡Heparin was furnished in generous quantities through the courtesy of both The Upjohn Co., Kalamazoo, Mich., and Hoffmann-La Roche, Inc., Nutley, N. J.

RESULTS

Effect of Sublingual Heparin and Treburon.—That heparin might possess an anticoagulant effect after sublingual absorption was suggested by Litwins and associates.[6] Therefore both heparin and Treburon were administered by this route either in the form of tablets, or cotton pledgets, or sugar cubes soaked with a solution containing the desired dose. Presented in highly condensed form, the results of some 200 trials with 70 patients showed no consistent prolongation of clotting time after one, three, five, seven, or twelve hours with doses of 140, 280, or 560 mg. of heparin or of 250, 500, 750, or 1,000

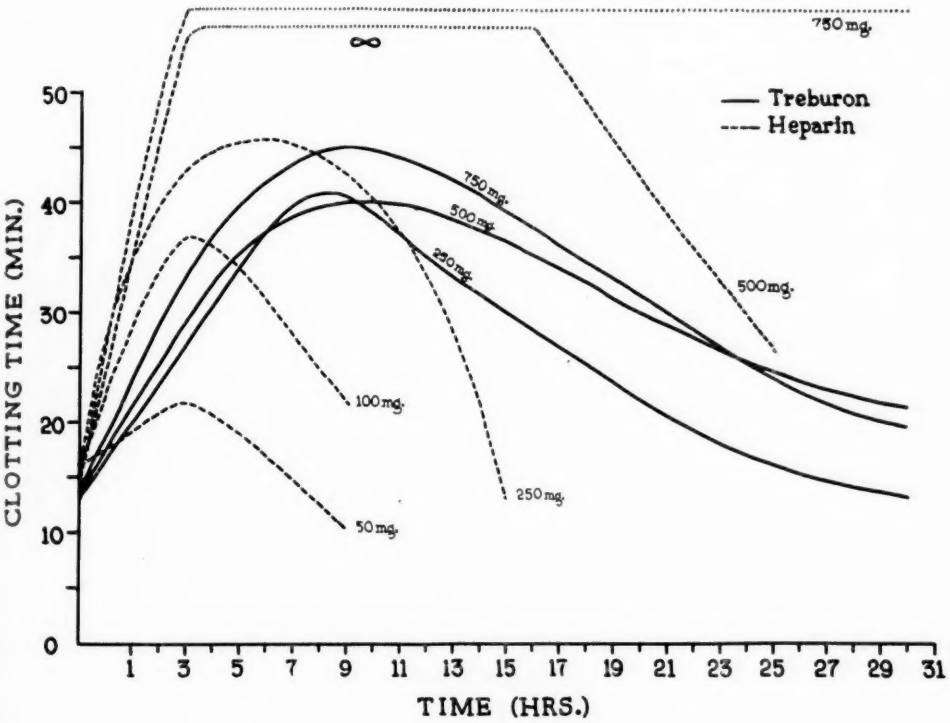


Fig. 1.

mg. of Treburon. One of 12 patients receiving 250 mg. Treburon and 2 of 14 receiving 750 mg. Treburon and one of 12 patients receiving 140 mg. heparin and one of 10 patients receiving 560 mg. heparin had a prolongation equal to doubling of the control values. One patient had a significant prolongation from nine minutes to twenty-three minutes one hour after receiving 250 mg. Treburon sublingually and to twenty-two minutes one hour after receiving 140 mg. heparin sublingually (the clotting time was twelve minutes after four hours).

Effect of Micronized Treburon by Inhalation.—The delivery of highly pulverized therapeutic agents to the vascular epithelium of the naso-pulmonary bed is an effective route of administration for certain antibiotic agents resulting particularly in rapid and high blood levels of penicillin.[7] This route was also explored in an attempt to make the heparinoid agents effective by a nonparenteral route. A micronized preparation of Treburon* was administered through a commercial powder inhalator† in doses of 250 and 500 mg. to a total of 9 patients. The clotting time two hours after administration showed no prolongation over the control values.

Effect of Intramuscular Treburon Compared with Heparin.—The effects of heparin are well established. Certain control studies, however, by the techniques of this laboratory were necessary for purposes of comparison with Treburon. Thus heparin was administered at levels of 50, 100, 250, 500, and 750 mg. to from 3 to 8 patients at each dose, and the average clotting times at various time intervals are recorded in Fig. 1. Similarly, 250, 500, and 750 mg. of Treburon were administered intramuscularly to from 13 to 18 patients

TABLE I. EFFECT OF TREBURON* IN HEPATIC AND RENAL DISEASE

PATIENT	DIAGNOSIS	LABORATORY DATA	CONTROL	CLOTTING TIME (MIN). AFTER 2 HR.	5 HR.	11-12 HR.	21-22 HR.
O.	Laennec's cirrhosis	I I 74 A/G 1.8/4.1 thymol turb. 11 ceph. flocc. 4+	12	51	66	18	
W.	infectious hepatitis	I I 40	17	41	38	18	
S.	subacute cirrhosis	I I 37 ceph. flocc. 4+ A/G 3.0/2.3	17	35	54	22	8
C.	cirrhosis	BSP—30% retention ceph. flocc. 2+ A/G 4/2.4	9	36	40	20	12
E.	metastatic liver disease	A/G 3.2/2.0 ceph. flocc. neg. thymol turb. 2	10	22	32	29	17
W.	chronic hepatitis	I I 13 ceph. flocc. 2* A/G 3.6/3.4 thymol turb. 7	22	63	65	28	12
J.	nephrosclerosis	NPN—180	5	24	25	11	
G.	chronic pyelonephritis	NPN— 62	10	77	74	30	18
B.	nephrosclerosis	NPN— 80	16	27	29	14	
R.	terminal Kimmelstiel-Wilson Disease	NPN— 80	19	40	48	36	11
E.	chronic glomerulonephritis	NPN—246	27	45	32	29	17
R.	chronic pyelonephritis	NPN— 60	11	18	29	11	

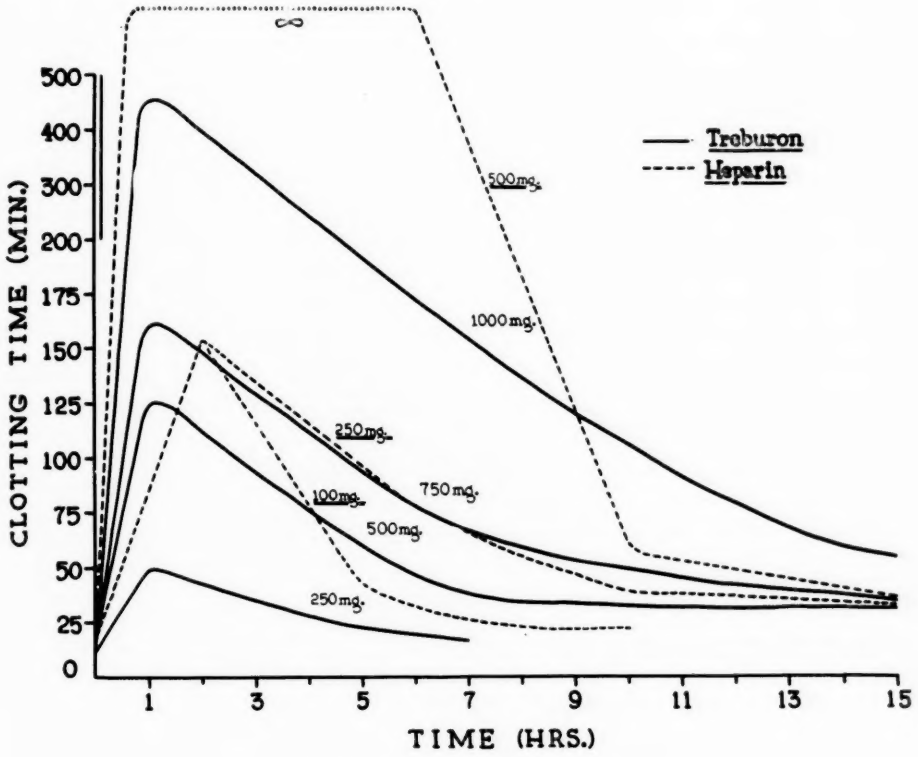
I I — Icterus index Thymol turb — thymol turbidity BSP — Bromsulfalein
A/G — Albumin/Globulin Ceph. flocc. — cephalin flocculation NPN — Nonprotein nitrogen

*A standard dose of 250 mg. i.m. was given to each patient.

*Prepared by Hoffmann-La Roche, Inc., Nutley, N. J.

†Penlators were furnished through the courtesy of Lederle Laboratories, Pearl River, N. Y.

at each dose level, and the results are also summarized in Fig. 1. The effect of intramuscularly-administered Treburon was detectable as early as fifteen minutes. After this time and following 250 mg. Treburon the clotting time increased from a control value of twelve minutes to fifteen minutes; following 500 mg. Treburon the value was eighteen minutes. In one hour the prolongation was to twenty minutes and twenty-four minutes, respectively. These compared with a prolongation to twenty-six minutes one hour after the intramuscular administration of 100 mg. heparin.



I.V. ADMINISTRATION

Fig. 2.

Effect of Intravenous Treburon Compared with heparin.—Heparin was administered intravenously to 3 patients at each level in doses of 100, 250, and 500 mg., and the resulting clotting times are plotted in Fig. 2. Similarly, 250, 500, 750, and 1,000 mg. of Treburon were given to 6 patients at each level, and the average clotting times are given in Fig. 2.

Effect of Treburon in Hepatic and Renal Disease.—To delineate the clinical limitations of Treburon therapy beyond the obvious contraindications, an evaluation of the effect in patients with varying degrees of hepatic and renal disease was obtained. Since 250 mg. of Treburon intramuscularly produced

a significant anticoagulant response (Fig. 1), this dose was administered to each patient. In Table I are given the protocols of these studies as well as the pertinent laboratory data and diagnoses.

In a similar manner, patients with hepatic and renal diseases were given 250 mg. of Treburon intramuscularly twice daily, and their response was compared with patients who received this therapy for therapeutic purposes. Whereas a very uniform and adequate anticoagulant response was obtained in patients with thrombophlebitis and myocardial infarction,[3] considerable variation with frequent unduly prolonged clotting times were observed in patients with the disorders given in Table I. Particularly in cases of reduced renal function with nitrogen retention was variability encountered, and onset of mild to moderate purpura was observed even with clotting times only slightly prolonged. This is probably related to the increased purpuric tendency noted in individuals with nitrogen retention.[8] Furthermore, when Treburon given for 6 to 8 doses of 250 mg. twice daily was discontinued, the clotting time would remain prolonged for as long as three to five days, whereas it returned to normal almost immediately in patients with adequate renal function. This is in accord with the observation of a renal excretory mechanism for Treburon[1] which parallels the route of excretion of heparin.[9]

Effect of Treburon on Prothrombin Time.—It has been reported that heparin[9] and Treburon[1, 2] affect the prothrombin time as determined by conventional methods. It is apparent that these agents affect the assay because of their in vitro antithrombic and antithromboplastic actions and not because of any in vivo influence. Using a technique founded upon a routine of diluting out plasma inhibitors and accelerators,[5] it was determined that Treburon had no effect on prothrombin concentrations (Table II).

TABLE II. EFFECT OF TREBURON ON PROTHROMBIN TIME

	WHOLE PLASMA (SEC.)	DILUTED PLASMA (SEC.)
Before Treburon	18.5	24.5
After Treburon	30.0	24.3

A protocol taken from a patient before and four hours after 500 mg. Treburon given intramuscularly.

DISCUSSION

The new agent Treburon has been demonstrated to resemble heparin closely in regard to physiologic activity and toxicity.[1, 2] In animal studies it possessed one-fourth to one-half the anticoagulant activity of heparin. The present studies in man point up a significant advantage from a therapeutic standpoint which results on intramuscular administration. In intravenous tests the duration of therapeutic effect (clotting time twice that of normal) was maintained with 250 mg. Treburon for five hours and with 500 mg. for at least fifteen hours, whereas 100 mg. of heparin maintained a similar action somewhat more than ten hours and 250 mg. of heparin was effective for fourteen hours. Thus intravenous heparin was perhaps two to three times more effective than Treburon on a weight basis. However, given intramuscularly

while 250 mg. of heparin had a therapeutic effect for only sixteen hours, Treburon in a dose of 250 mg. was effective for roughly twenty-four hours, and with 500 mg. most patients still showed therapeutic prolongation of the clotting time for twenty-four to thirty hours. Thus a single intramuscular injection daily of 250 mg. of Treburon gave a significant anticoagulant action for a longer period than the same dose of heparin and for almost a full twenty-four hour period. It might be added that several additional observations favor Treburon over heparin; in intramuscular administration with heparin occasional local pain and hematoma were obtained, the latter especially frequent in doses of 250 mg. or higher. With Treburon no *local* reaction of any kind has been observed.[2] However, several serious toxic phenomena following repeated administration of Treburon have been observed, and they will be treated in the report dealing with the clinical usefulness of Treburon.

It would appear that aside from some greater variation in hypocoagulability in patients with renal and liver disease, single 250 mg. doses of Treburon were well tolerated. Some tendency to accumulation was noted, especially in instances of reduced renal clearance, and this appears related to the mechanism of excretion through the kidneys. An augmented bleeding tendency in uremics given anticoagulants as heparin or Treburon with ordinarily "safe" levels of hypocoagulability may be related more to an increased fragility phenomena in these individuals rather than any intravascular effect.

Apparently contrary to a previous report,[6] heparin is not consistently absorbed from the sublingual route. Similarly, the heparinoid agent, Treburon, is not consistently effective if administered sublingually. The ineffectiveness of sublingual heparin has been recorded by others,[10, 11] and it has been indicated that sublingual Treburon had no effect on induced hyperlipemia.[12]

CONCLUSIONS

Heparin and Treburon were administered to 200 hospitalized patients. The sublingual administration of both these agents produced only occasional prolongation of the clotting time of the blood. The inhalation of micronized Treburon powder was ineffective.

On a weight basis, Treburon was one-half to one-third as effective compared with heparin by the intravenous route. However, single large intramuscular injections of Treburon produced significant hypocoagulability in a more reproducible manner for twenty-four hour periods than did large doses of heparin. At levels of 250 mg. heparin was effective for some sixteen hours, Treburon for twenty-four hours. No local reactions at the site of injection occurred with Treburon, whereas hematoma and pain resulted with large doses of heparin. However, certain other systemic toxic phenomena have been observed.

In patients with renal and liver disease fairly uniform hypocoagulability resulted after a single dose of Treburon. However a variable response, purpura without marked hypocoagulability and tendency to accumulation was observed after multiple doses of Treburon in patients with reduced renal function.

We are grateful to Mr. Ray Hartwig for technical assistance and to Dr. Robert Stragnell for the prothrombin estimations.

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BLOOD SERUM MAGNESIUM IN PORTAL CIRRHOSIS AND DIABETES MELLITUS

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THE importance of magnesium for normal body metabolism has been demonstrated repeatedly. More specifically, it is known to be related in some fashion to carbohydrate[1-6] and fat[7] metabolism. What role magnesium plays in the etiology of disease and, conversely, what effects disease per se has upon the magnesium ion are still in the early stages of clarification.

This study was made to supplement previous papers in the literature which have demonstrated electrolyte disturbances in the blood serum of patients with portal cirrhosis[8] and diabetes mellitus[9] by determining whether or not such pathologic states might also be accompanied by alterations of the blood serum magnesium concentration.

MATERIALS AND METHODS

The control group consisted of 17 normal adult males ranging from 20 to 68 years of age. An attempt was made to have selective decade grouping so that the age range and distribution of the controls would correspond fairly well with that of the abnormal groups. The latter groups consisted of 23 adult males with portal cirrhosis and 101 adult males with diabetes mellitus. All of the diabetic patients were reasonably well nourished, and with one exception were adequately controlled by diet (31 cases) or diet plus NPH insulin (70 cases). The criterion for control was the presence of no more than a one plus quantity of sugar in an early morning specimen of urine. Blood sugar and hepatic function tests were not carried out in the diabetic group at the time of this study, as the diagnosis had been made previously in both series by clinical and laboratory means.

Blood serum magnesium determinations were made in duplicate by the method of Simonsen and co-workers.[10] As reported previously, no distinction was made between fasting and nonfasting states.[11]

RESULTS

In the control group the mean blood serum magnesium from seventeen fasting and nonfasting adults was 1.82 ± 0.12 meq. per liter. In the group of patients with portal cirrhosis the mean serum magnesium was 1.49 ± 0.21 meq. per liter (Table I). This difference on comparison with the control group was statistically significant (Table II). Of the 101 patients with diabetes mellitus, 97 were grouped for purposes of statistical analysis on the basis of their daily insulin requirement (Table I). Four of the patients were excluded (Table IV) as three had an associated renal impairment and the fourth had been admitted to the hospital a few days previously in diabetic acidosis. The

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TABLE I. BLOOD SERUM MAGNESIUM (MEQ./LITER) IN NORMAL, CIRRHOSIS AND DIABETES MELLITUS

		DIABETES MELLITUS			
NORMAL	CIRRHOSIS	NO INSULIN	<20 U. INS.	20-40 U. INS.	>40 U. INS.
1.67	1.10	1.47	1.53	1.30	1.42
1.71	1.21	1.54	1.55	1.35	1.43
1.73	1.26	1.57	1.56	1.42	1.45
1.73	1.27	1.60	1.64	1.50	1.47
1.75	1.28	1.61	1.65	1.52	1.49
1.77	1.30	1.63	1.65	1.54	1.52
1.79	1.38	1.64	1.68	1.58	1.53
1.81	1.41	1.72	1.79	1.58	1.55
1.81	1.43	1.73	1.82	1.61	1.56
1.82	1.44	1.76	1.88	1.62	1.61
1.82	1.50	1.77	2.13	1.63	1.62
1.83	1.54	1.78		1.63	1.64
1.84	1.54	1.83		1.66	1.64
1.84	1.55	1.85		1.66	1.65
1.91	1.55	1.87		1.67	1.65
1.99	1.57	1.88		1.67	1.65
2.16	1.61	1.88		1.70	1.69
	1.63	1.89		1.71	1.70
	1.70	1.92		1.73	1.72
	1.70	1.93		1.74	1.73
	1.74	1.94		1.77	1.73
	1.74	1.96		1.79	1.73
	1.81	1.97		1.81	1.74
		2.00		1.81	1.78
		2.05		1.81	1.80
		2.12		1.83	1.84
		2.14		1.84	1.85
				1.87	1.86
					1.87
					1.87
					1.89
$\bar{x}$ 1.82 ± .12	1.49 ± 0.20	1.82 ± 0.18	1.72 ± 0.18	1.66 ± 0.15	1.67 ± 0.14

mean serum magnesium value in the 27 diabetic patients controlled by diet alone was 1.82 ± 0.18 meq. per liter; for eleven patients requiring less than 20 units of insulin daily, 1.72 ± 0.18 meq. per liter; for twenty-eight patients requiring from 20 to 40 units of insulin daily, 1.66 ± 0.15 meq. per liter; and for thirty-one patients requiring more than 40 units of insulin daily, 1.67 ± 0.14 meq. per liter. Patients with diabetes mellitus requiring 20 units or more

TABLE II. STATISTICAL COMPARISON OF NORMAL TO CIRRHOSIS OF THE LIVER AND TO DIABETES MELLITUS

GROUP	NO.	MAGNESIUM MEQ./LITER	t	P
1. Normal	17	1.82 ± 0.12	6.53	<0.01
Cirrhosis	23	1.49 ± 0.20		
2. Normal	17	1.82 ± 0.12	0.13	>0.10
Diabetics requiring no insulin	27	1.82 ± 0.18		
3. Normal	17	1.82 ± 0.12	1.93	>0.10
Diabetics requiring <20 u. insulin	11	1.72 ± 0.18		
4. Normal	17	1.82 ± 0.12	3.98	<0.01
Diabetics requiring 20 to 40 u. insulin	28	1.66 ± 0.15		
5. Normal	17	1.82 ± 0.12	3.97	<0.01
Diabetics requiring >40 u. insulin	31	1.67 ± 0.14		

of insulin showed a statistically significant difference of the serum magnesium on comparison with the control group (Table II).

Finally, the serum magnesium values of the four groups of diabetes mellitus were compared. A definite statistically significant difference was found between the groups requiring more than 20 units of NPH insulin and that controlled by diet alone (Table III).

TABLE III. STATISTICAL COMPARISON OF DIABETICS REQUIRING AND NOT REQUIRING INSULIN

GROUP	NO.	MAGNESIUM MEQ./LITER	t	P
1. Diabetics, no insulin required	27	1.82 ± 0.18	1.58	>0.10
Diabetics, requiring <20 u.	11	1.72 ± 0.18		
2. Diabetics, no insulin required	27	1.82 ± 0.18	3.65	<0.01
Diabetics, requiring 20 to 40 u.	28	1.66 ± 0.15		
3. Diabetics, no insulin required	27	1.82 ± 0.18	3.63	<0.01
Diabetics, requiring >40 u.	31	1.67 ± 0.14		

The patient with diabetic acidosis (Table IV) had a lower blood serum magnesium value than the controlled diabetic patients. In the three patients with renal dysfunction associated with the diabetes mellitus, the blood serum magnesium values were elevated.

DISCUSSION

The serum magnesium value for the control group was lower than that reported previously for a group of 92 normal males (1.94 ± 0.21 meq. per liter).[11] This difference is probably a seasonal variation and may be related to vitamin C or D.[12] The higher value was obtained during the summer and the lower during the winter season.

The present study revealed that in portal cirrhosis and moderately severe diabetes mellitus a significantly low serum magnesium may be found. The severity of the diabetes mellitus appears to be related to a degree to the reduction of the serum magnesium.

An explanation for the low serum magnesium in portal cirrhosis and diabetes mellitus is not readily apparent. The literature is sparse regarding the metabolism of magnesium in man. However, a low serum magnesium may be related to a decreased dietary intake, a decreased gastrointestinal absorption, an increased intracellular shift of magnesium ions, or an increased urinary or intestinal excretion. The two chief sources of dietary magnesium are milk and vegetables, green vegetables being particularly high in magnesium, as the latter occurs in chlorophyll. The average diet (3,000 calories) contains approximately 0.34 Gm. (0.17 to 0.67 Gm.) of magnesium.[13] Tibbitts and Aub[14] showed that 0.22 Gm. per day of magnesium would cause a positive magnesium balance in adults on a neutral diet, whereas Brull found that 0.0034 Gm. of magnesium per kilogram of body weight per day was necessary for positive balance.[15] It was also found that 60 per cent of the magnesium excreted by the body was found in the feces.[14]

The patients with portal cirrhosis were also chronic alcoholics and had had an inadequate nutritional intake for three to five months. This may account for the low serum magnesium values.

TABLE IV. BLOOD SERUM MAGNESIUM IN DIABETES MELLITUS (UNCONTROLLED OR COMPLICATED)

UNITS OF INSULIN	CONDITION	MAGNESIUM (MEQ./L.)
1. 0 unit	Diabetic acidosis	1.27
2. 22 units	Diabetes and renal impairment	2.47
3. 0 unit	Diabetes and renal impairment	2.73
4. 20 units	Diabetes and renal impairment	2.31

There was no restriction of diet in patients with controlled diabetes mellitus. It would be unlikely that the low serum magnesium would be related to an inadequate nutritional intake. It is well known that magnesium is a cofactor in the metabolism of carbohydrate[16, 17] and the low serum magnesium may be related to the severity of impaired carbohydrate utilization. There may also have been an increased urinary excretion of magnesium in relation to the glycosuria, for it is known that the serum magnesium is related to the urinary excretion of magnesium.[18, 19] The three diabetes mellitus patients with an associated renal dysfunction had an elevated serum magnesium.[20]

SUMMARY

Blood serum magnesium was studied in 23 patients with portal cirrhosis and in 101 patients with diabetes mellitus. The group of patients with portal cirrhosis had the lowest serum magnesium, and in the patients with diabetes mellitus there appears to be a correlation between the severity of the disease process and the reduction in level. Increased values were found in the patients with diabetes mellitus plus renal dysfunction. The possible implications of the low serum magnesium are discussed.

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FURTHER OBSERVATIONS ON THE USE OF THE URINARY PIGMENT-CREATININE RATIO FOR THE MEASUREMENT OF BASAL METABOLIC RATE

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SINCE the introduction of the method of measuring the basal metabolic rate by determination of the urinary pigment-creatinine ratio[1, 2] there have been numerous inquiries from other investigators who have had difficulty in measuring the pigment excretion. This difficulty stems from the fact that the measurement is determined by the per cent light transmission of the urine at wave length 420 μ on a Coleman Junior spectrophotometer, and the Coleman light transmission figure is then converted to a corresponding reading obtained from the dial reading of a Klett photometer, since the original determinations of pigment concentration were obtained on this instrument. The Klett reading is thus the figure used in the equations:

Male	B.M.R. = 57.0 + 0.25 P/C
Female	B.M.R. = 54.7 + 0.22 P/C

Since the conversion table of Coleman to Klett readings is not generally available, a Klett-Coleman equation was derived by fitting, by the method of least-squares, a straight line equation to nine pairs of Klett-Coleman readings:

$$\text{Klett} = 1739.05 - 867.75 \quad (\log \text{ Coleman})$$

Thus it is seen that the figure for the pigment concentration in the formula for calculating the basal metabolic rate is obtained by first converting the Coleman light transmission reading to a logarithm and then substituting the derived Klett reading (pigment concentration) in the appropriate equation for males or females.

It is necessary to repeat that this method of calculating metabolic rate is not valid in conditions in which the urine is abnormally colored, namely, after the ingestion of foods such as beets and rhubarb, large quantities of riboflavin, or organic dyes. The test cannot be used in cases of severe liver damage or renal disease with nitrogen retention.

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THE QUANTITATIVE MEASUREMENT OF VIBRATORY PERCEPTION IN SUBJECTS WITH AND WITHOUT DIABETES MELLITUS

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INSULIN, antibiotics, and adequate nutrition have extended markedly the life expectancy of the patient with diabetes mellitus. This prolongation of life, however, is marred by the development of retinopathy, nephropathy, neuropathy, and arteriosclerosis involving the lower extremities, the kidneys, the heart, and other parts of the vascular tree. Although it has been common knowledge that the presence of diabetes is characterized by a marked increase in the incidence of vascular degeneration, the true magnitude of the problem did not become apparent until 1947.[1] At that time Dolger demonstrated that not one of 200 patients, whom he examined regularly, escaped the development of retinal hemorrhages and/or other manifestations of vascular damage. These findings were soon confirmed by White who found vascular damage in 92 per cent of children with diabetes who survived for twenty or more years after onset of the disorder.[2]

In contrast with the numerous reports on the extremely high incidence of vascular damage, there are relatively few studies on the incidence of neuropathy in patients with diabetes mellitus. Whereas some have claimed that neuropathy is relatively rare in such patients,[3-8] others have reported an incidence of from 30 to 90 per cent.[9-13] This discrepancy can be attributed in large measure to the diagnostic criteria employed by different investigators. Some insist that the presence and persistence of most of the classical signs and symptoms (pain, tenderness, paresthesias of greatest intensity at night, muscle cramps, loss of vibratory perception, loss of tendon jerks, etc.) are essential to the diagnosis of peripheral neuropathy, whereas others have accepted some single symptom or sign such as diminution of vibratory perception as diagnostically significant.

In 1922 Williamson first emphasized that impairment of vibratory perception may be one of the first signs of peripheral neuropathy in the patient with diabetes.[14] Subsequently, numerous attempts have been made to utilize some quantitative measure of this impairment, the most recent being that of

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Collens, Zilinsky, and Boas,[12] who reported that approximately 90 per cent of patients with diabetes exhibit a deviation from "normal." In order to evaluate the significance of data such as that reported by Collens and associates, it is essential to determine the factors that may influence the measurement of vibratory perception. Toward that end the vibratory perception was studied in a carefully selected group of subjects without diabetes as well as in a series of patients with diabetes.

METHOD

All subjects were ambulatory and were selected to exclude those with any disorder, other than diabetes, which likewise is known to impair vibratory perception. Among those excluded were patients with any evidence or history of a previous head injury, hypothyroidism, syphilis, lymphopathia venereum, pernicious anemia, malignant neoplasms, active tuberculosis, febrile disorders, malnutrition, alcoholism, lead intoxication, spinal osteoarthritis and radicular symptoms, thromboangiitis obliterans, intermittent claudication, other peripheral vascular diseases, and nondiabetic neuropathies. Further, no subject with lesions of the legs, amputation of an extremity, blindness, or deafness was included in this study.

Vibratory perception was measured by means of an inaudible electromagnetic vibrator with a frequency of 120 per second which was activated by a 60-cycle alternating current, the voltage being controlled with a variable transformer. The vibrator weighed 330 Gm. The tactor of the vibrator ended in a firm rubber cylinder whose flat contacting surface was 14 mm. in diameter, a size which permitted not only a better spread of vibration through the tissues,[15] but also a good correlation between the energy consumption of the tactor and the threshold of vibratory perception.[16] The vibrations produced consisted of two components, the amplitude of one being so small that it could be measured only with difficulty. The amplitude of the chief component of vibration was a function of the square of the voltage and consequently the threshold of vibratory perception could be expressed in units of "volts^2".

The tests were performed in a quiet room with the patient relaxed on a bed or examining table. The suggestibility and attentiveness of the patient were tested before any measurements were made by noting the minimal vibration that he could perceive over the hypothenar eminence and then determining whether or not the perception was still present when the current was turned off and on without the patient's awareness. Subjects who reported the perception of vibration when the current was turned off were excluded from the study.

Since it has been demonstrated that the fingers and toes are most sensitive to vibratory stimulation,[17-19] and since the neuropathies of diabetes mellitus may assume a glove and stocking distribution, the plantar surfaces of the great toes were selected as the sites for measurement of the threshold of vibratory perception. Each subject was tested by allowing the tactor to rest perpendicularly to the palmar surface of the index finger or to the plantar surface of the great toe, about ¼ inch proximal to the end of the nail. Thereby the effects of changes in the pressure applied to the part were minimized. The strength of the current activating the vibrator was then gradually increased from 0 volts until the subject reported his perception of the vibration. A minimum of three measurements was made for each digit, and the average voltage noted. From twenty to forty seconds were permitted to elapse between successive measurements. Neither the speed with which the subject's threshold for the perception of vibration was approached nor the removal and reapplication of the vibratory stimulus after each response was found to exert any significant influence upon the measurements. The mean value of these consecutive measurements was used as an estimate of the vibratory perception threshold (VPT). In every instance in which data were obtained and used in this study, three consecutive values showed no significant fluctuation about the mean.

TABLE I. RELATION OF VIBRATORY PERCEPTION THRESHOLD TO AGE OF SUBJECTS

			NONDIABETIC					DIABETIC				
SEX	SIDE	EXTREMITY	NO.	MEAN AGE (YEARS)	MEAN VPT LOG (VOLTS^2)	b	t	NO.	MEAN AGE (YEARS)	MEAN VPT LOG (VOLTS^2)	b	t
Male	Right	Index finger	68	31.0	1.7743	0.0112	12.68*	37	31.7	1.9124	0.0137	6.99*
		Great toe	68	31.0	1.9426	0.0181	13.93*	37	31.7	2.3654	0.0214	6.37*
	Left	Index finger	62	31.9	1.7546	0.0115	8.57*	28	35.8	2.0116	0.013	4.29*
		Great toe	62	31.9	1.9446	0.0153	8.44*	28	36.7	2.4758	0.0186	5.71*
Female	Right	Index finger	68	36.3	1.7986	0.0071	8.07*	70	42.5	2.0254	0.0089	6.29*
		Great toe	68	36.3	1.9299	0.0139	10.22*	70	42.5	2.4218	0.0204	9.16*
	Left	Index finger	65	37.4	1.7754	0.0089	11.99*	58	47.1	2.038	0.0073	3.66*
		Great toe	65	37.4	1.9284	0.0151	12.7*	61	46.5	2.5118	0.0196	19.0*
Both	Right	Index finger	136	33.6	1.7368	0.0097	13.9*	107	38.8	1.9863	0.0103	9.0*
		Great toe	136	33.6	1.9362	0.0157	16.66*	107	38.8	2.4023	0.0198	10.7*
	Left	Index finger	127	34.7	1.7652	0.0098	13.0*	86	43.5	2.0294	0.0083	4.98*
		Great toe	127	34.7	1.9363	0.0149	13.0*	89	43.5	2.5005	0.0184	7.03*

b = Coefficient of linear regression of vibratory perception threshold [log (volts^2)] on age.
t = Critical ratio of coefficient of regression to its standard error.
*Significant at 0.1 per cent level.

The threshold for the perception of the vibratory stimulus applied to the index finger and great toe was measured in 102 patients with diabetes mellitus and in 136 subjects without diabetes. In some instances measurements were made only on the right side. The two groups were essentially similar with respect to distribution by age, there being no significant difference between diabetic and nondiabetic men ($P = 0.9$), diabetic and nondiabetic women ($P = 0.9$), nor between nondiabetic men and women ($P = 0.4$) or diabetic men and women ($P = 0.7$).

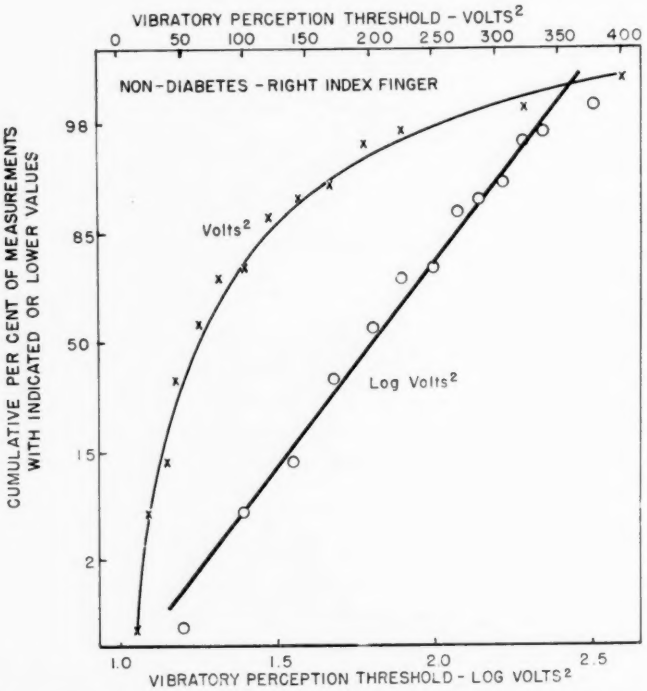

Fig. 1A.—Frequency distribution of probit plots of the vibratory perception thresholds of the right index finger in subject without diabetes expressed as volts² and logarithms of volts.² The smooth lines have been fitted to the respective data by inspection and indicate that the systematic deviations are removed by logarithmic transformations.

RESULTS

Expression of Data.—Before proceeding to determine the influence of various factors on the vibratory perception threshold, it became necessary to establish the most efficient manner in which the experimental data could be expressed. Since conventional statistical procedures apply most efficiently to data which are distributed normally, the nature of the distribution of the vibratory perception thresholds for the different groups of individuals was determined by plotting on probability paper.

When the cumulative percentage frequency of a variable is plotted on probability paper against the units of that variable, the plot is a straight line when the distribution is normal. Typical results of such plots are illustrated in Fig. 1, which demonstrates the distribution frequency for measurements of the VPT when the vibratory stimulus was applied to the right index finger of

subjects without diabetes and the right great toe of subjects with diabetes. When the data representing the vibratory perception threshold (volts^2) were expressed arithmetically, a straight line was not obtained, i.e., the values did not group themselves in the form of a normal distribution curve. When the same procedure was repeated but the vibratory perception threshold was expressed exponentially in terms of the Briggsian logarithm of the volts^2 (2 log

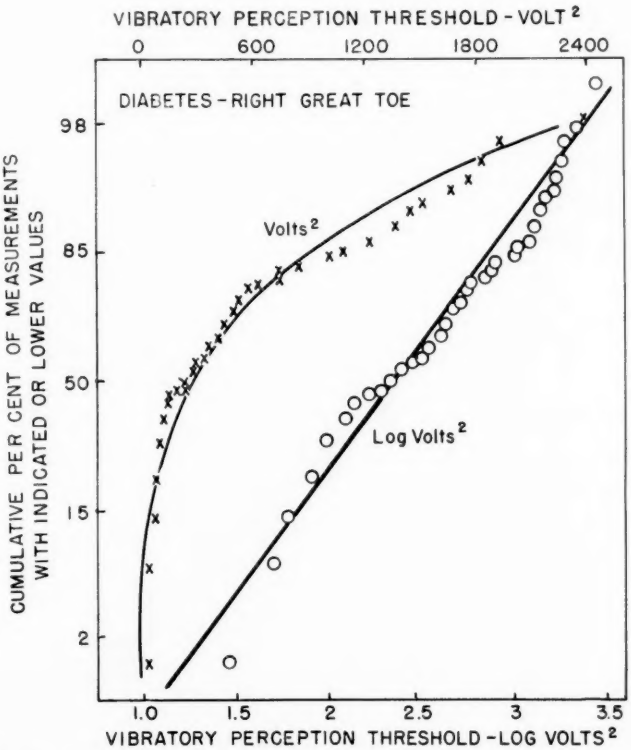

Fig. 1*B*.—Frequency distribution of probit plots of the vibratory perception thresholds of the right great toe in patients with diabetes expressed as volts^2 and logarithms of volts.^{2} The smooth lines have been fitted to the respective data by inspection and indicate that the systematic deviations are removed by logarithmic transformations.

volts), the data were fitted well by a straight line without the systematic deviation characteristic of the arithmetic plots. Straight lines were obtained also for the vibratory perception thresholds expressed as log volts^2 when the other sites were used in both diabetic and nondiabetic subjects.

Accordingly, in order to compare efficiently the VPT of different subjects, the data must be expressed in terms of the logarithm of the units (log volts^2) rather than in terms of the arithmetic values.

Age.—A linear relationship between the age of the subjects and the threshold for vibratory perception was observed (Fig. 2). Thus, irrespective of the site of measurement, the coefficient of linear regression of the VPT on

age was statistically highly significant (Table I). Consequently, in subsequent analyses of the influence of various factors on the VPT, analysis of covariance was utilized so that the effect of age could be kept constant.

TABLE II. SIGNIFICANCE OF DIFFERENCES BETWEEN VIBRATORY PERCEPTION THRESHOLDS (VPT) AND BETWEEN REGRESSION COEFFICIENTS OF VPT ON AGE

				NON-DIABETIC								DIABETIC							
				Male				Female				Male				Female			
				Right		Left		Right		Left		Right		Left		Right		Left	
				Finger	Toe	Finger	Toe	Finger	Toe	Finger	Toe	Finger	Toe	Finger	Toe	Finger	Toe	Finger	Toe
NON-DIABETIC	Male	Right	Finger		*** 21.92	0.83		0.65				*** 107.58							
			Toe	*** 19.93			1.08		* 5.39				*** 46.17						
		Left	Finger	0.30			*** 31.48			1.06				*** 14.5					
			Toe		1.76	2.91					** 5.78				*** 32.57				
	Female	Right	Finger	** 10.65					*** 40.43	1.24						*** 29.4			
			Toe		* 4.98			*** 15.74			0.16						*** 48.92		
		Left	Finger			2.75		1.72			*** 12.97							*** 19.91	
			Toe				0.01		0.36	*** 22.66									*** 41.46
DIABETIC	Male	Right	Finger	1.78									*** 67.13	0.50		0.14			
			Toe		1.03							3.26			0.01		3.84		
		Left	Finger			0.29						0.39			*** 18.64			* 6.95	
			Toe				0.58						2.24	0.91					2.03
	Female	Right	Finger					1.27				3.56					*** 49.63	1.19	
			Toe						* 6.51				0.06			*** 19.55			0.02
		Left	Finger							0.19				2.58		0.46			*** 50.13
			Toe								1.96				0.08		0.12	** 10.38	

The significance of difference between any two measurements of the vibratory perception threshold, adjusted for age by covariance, is indicated by the variance ratios to the right of the solid blocks. The significance of difference between any two regression coefficients is indicated by the variance ratios to the left of the solid blocks.

* = significant at 5 per cent level; ** = significant at 1 per cent level; *** = significant at 0.1 per cent level.

For example, the significance of the difference between the mean VPT of the right toe and right finger of nondiabetic men, after adjustment for age by covariance, is indicated by the variance ratio of 21.92, which is significant at the 0.1 per cent level. The significance of the difference between the regression coefficients of VPT of the right toe and right finger on the age of nondiabetic men is indicated by the variance ratio 19.93, which is significant at the 0.1 per cent level.

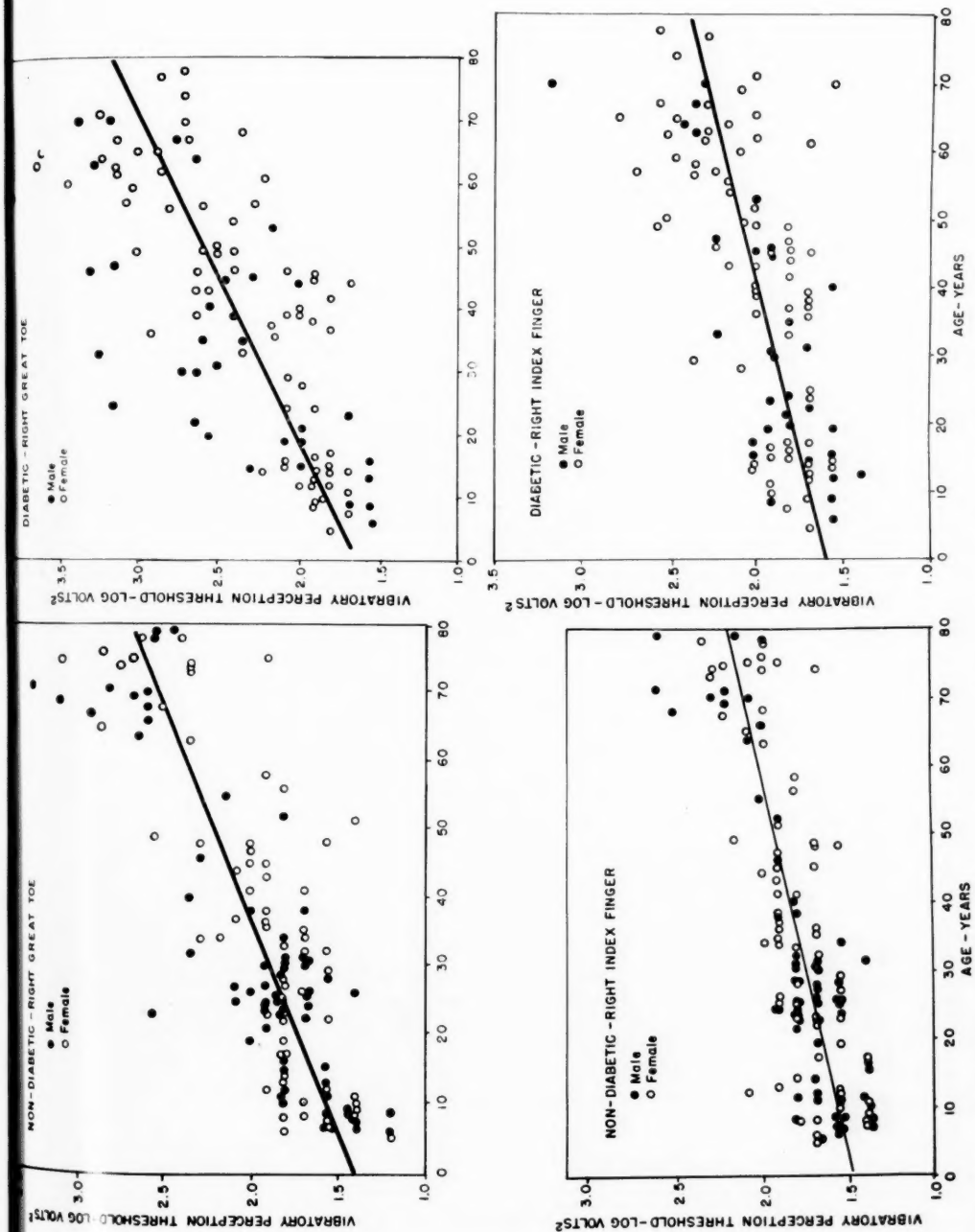
DIABETIC - RIGHT GREAT TOE
NON-DIABETIC - RIGHT GREAT TOE
DIABETIC - RIGHT INDEX FINGER
NON-DIABETIC - RIGHT INDEX FINGER
● Male
○ Female
VIBRATORY PERCEPTION THRESHOLD - LOG VOLTS²
AGE - YEARS

Fig. 2.—Vibratory perception thresholds as measured at different sites in subjects with and without diabetes. Each of the lines represents the linear regression of vibratory perception thresholds on age.

Site of Measurement.—For the reasons noted earlier, the palmar surface of the right and left index fingers and the plantar surfaces of the right and left great toes were selected as the sites of measurement. As is evident in Table II, analysis of covariance revealed no significant difference between either the adjusted means or the regression coefficients of VPT of the right and left index fingers or the right and left toes on age. Since the perception of vibration was essentially symmetrical, consideration of the role of various factors on vibratory perception may be confined to measurements made on one or the other side of the body.

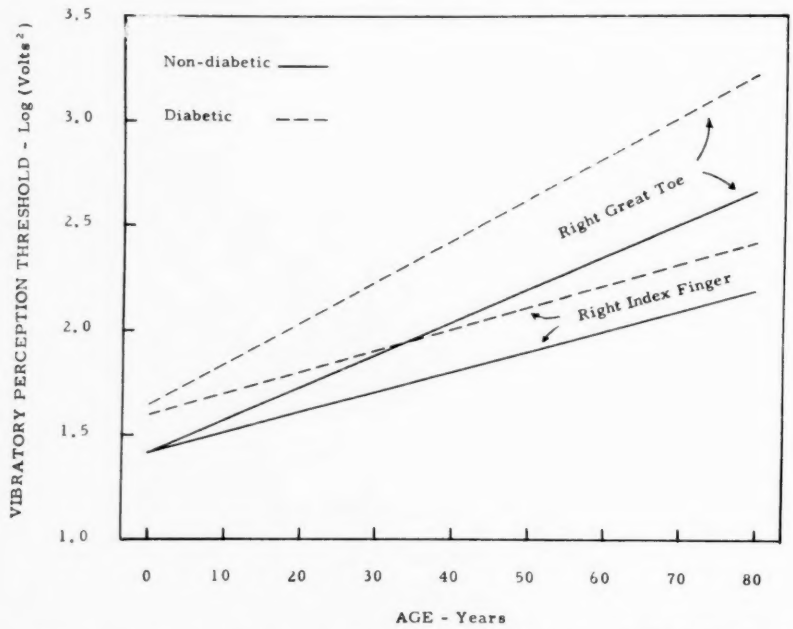

Fig. 3.—Linear regression of vibratory perception thresholds on age in subjects with and without diabetes.

A highly significant difference between the vibratory perception thresholds of the upper and lower extremities was noted in both diabetic and nondiabetic groups of subjects (Fig. 3). The threshold values for the great toes, after adjusting for age, were significantly greater than those for the index fingers (Table II). Likewise, the differences between the regression coefficients of the VPT of the index fingers and of the great toes on age were highly significant. Not only was the actual VPT higher in the toes than in the fingers but the rate of impairment of vibratory perception (slopes of linear regression of VPT on age) in the great toe was greater than in the index finger in all groups of subjects.

Sex.—In most instances no significant difference in the perception of vibration was noted between men and women. The mean VPT adjusted for age by covariance was essentially the same in men and women at all sites measured except the right and left great toes of the nondiabetic group, in

which a barely significant difference was noted (Table II). Likewise, the coefficients of linear regression of VPT on age were essentially the same for men and women except for measurements made on the right index finger ($P = 0.001$ to 0.01) and the right great toe ($P = 0.01$ to 0.05) of nondiabetic subjects. Accordingly, the rate of impairment of vibratory perception with age was of the same order in nondiabetic and diabetic men and women at all sites measured except for the right index finger and the right great toe of nondia-

TABLE III. SIGNIFICANCE OF DIFFERENCES BETWEEN VIBRATORY PERCEPTION THRESHOLDS (VPT) AND BETWEEN REGRESSION COEFFICIENTS OF VPT ON AGE OF NONDIABETIC AND DIABETIC SUBJECTS

			NONDIABETIC				DIABETIC			
			RIGHT		LEFT		RIGHT		LEFT	
			FINGER	TOE	FINGER	TOE	FINGER	TOE	FINGER	TOE
NONDIABETIC	RIGHT	FINGER		32.34*	2.73		79.64*			
		TOE	34.71*			1.16		85.71*		
	LEFT	FINGER	0.80			24.77*			26.12*	
		TOE		1.88	12.05*					69.3*
DIABETIC	RIGHT	FINGER	1.29					85.38*		
		TOE		4.29			19.86*			0.03
	LEFT	FINGER			0.79		1.07			61.48*
		TOE				1.92		0.21	10.49*	

The significance of difference between any two measurements of the vibratory perception threshold, adjusted for age by covariance, is indicated by the variance ratios to the right of the diagonal line. The significance of difference between any two regression coefficients is indicated by the variance ratios to the left of the diagonal line.

*Significant at the 0.1 per cent level.

betic subjects where the rates were 1.6 and 1.3 times greater in men than in women. In spite of the aforementioned exceptions, the differences between men and women were so small as to make it permissible to group them together in subsequent analysis.

Presence of Diabetes Mellitus.—The presence of diabetes mellitus is associated in both men and women, with a highly significant increase in the threshold for vibratory perception at all sites measured (Tables II and III). The rates of impairment of vibratory perception, as calculated from the regression co-

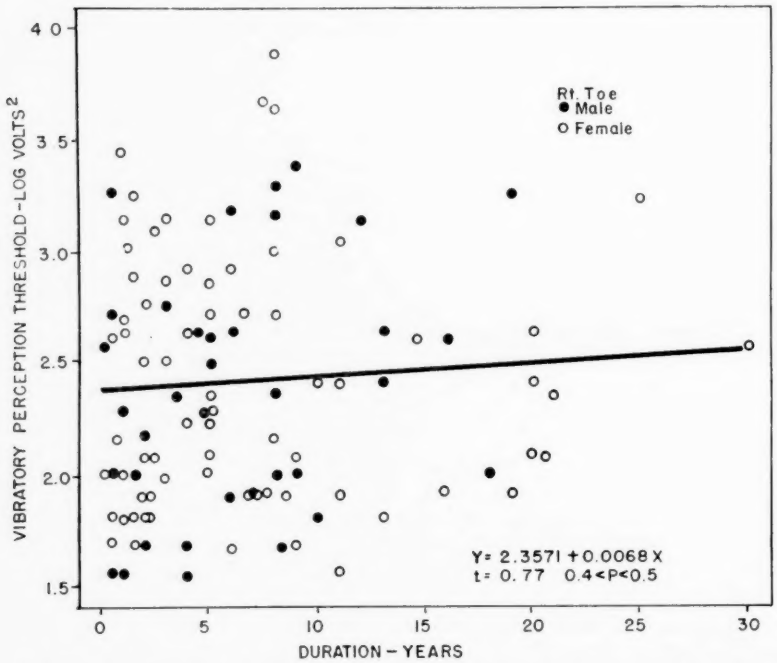

Fig. 4.—Relation between the vibratory perception threshold and duration of diabetes mellitus.

efficients, however, are not affected significantly by the presence of diabetes. Accordingly, the VPT of a patient with diabetes is the same as that of an *older* person without diabetes since the VPT, adjusted for age by covariance, is higher among men and women with diabetes whereas the *rate* of impairment of vibratory perception with aging is essentially the same as in the nondiabetic group (Fig. 3).

Since there is no significant difference between the regression coefficients of VPT on age of both groups of subjects, the mean of the coefficients can be utilized to calculate the age equivalence of any VPT. For example, the mean of the regression coefficients of VPT of the right index finger on the age of both groups is 0.01 so that

$$Y \text{ (nondiabetic)} = 1.4009 + 0.01 \text{ X nondiabetic and}$$
$$Y \text{ (diabetic)} = 1.5989 + 0.01 \text{ X diabetic,}$$

where Y = VPT and X = age. If Y (nondiabetic) = Y (diabetic), X (diabetic) + 19.8 = X (nondiabetic), i.e., the mean VPT of the right index fingers of patients with diabetes is the same as that of a group of nondiabetic subjects who are 19.8 years older. Similar calculations for the right toe yield a difference of 21.1 years. Accordingly, the mean VPT of patients with diabetes is similar to that of nondiabetic subjects approximately twenty years older.

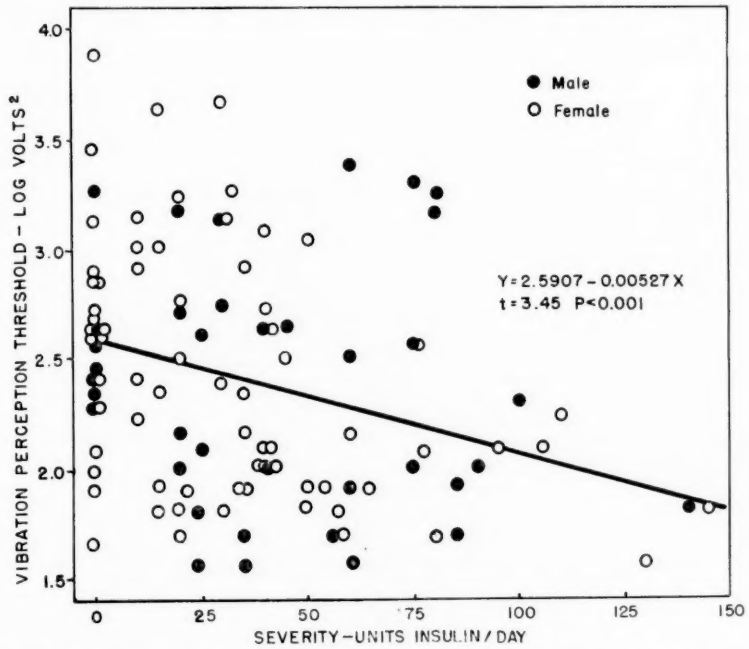

Fig. 5.—Relation between the vibratory perception threshold and severity of diabetes mellitus (insulin requirements).

Duration and Severity of Diabetes Mellitus.—The influence of the duration and the severity of the metabolic disorder on the threshold for vibratory perception was evaluated. For such purposes the duration of the diabetic syndrome was estimated by the years of known glycosuria or other pathognomonic signs and symptoms, whereas the severity of the existing syndrome was estimated on the basis of the total amount of insulin required per day for satisfactory regulation of the metabolic derangement. Even when no other variable is taken into consideration, there is no significant relationship between the threshold for vibratory perception and the duration of the syndrome, as is exemplified by Fig. 4, where the regression coefficient of the VPT of the right toes of men and women on the duration of the diabetes is 0.0068 ($t = 0.77$; $P = 0.4 - 0.5$).

As is illustrated in Fig. 5, a significant negative relationship between the VPT and the insulin requirements was noted ($b = 0.0053$; $t = 3.45$; $P < 0.001$). This observation could be interpreted to indicate that the milder

the metabolic disorder, the greater is the impairment of vibratory perception. Such an interpretation, however, is obviated by the fact that the older subjects have a greater degree of impairment in vibratory perception than do children but require less insulin for regulation of their metabolic derangement (Fig. 6).

In order to evaluate the relative effects of age, duration and severity on VPT, the multiple regression of VPT on the three variables was calculated, and revealed that neither duration nor severity played a significant role in the impaired vibratory perception of the diabetic subjects. Thus the partial regression coefficient of VPT on age (0.0216) was found to be highly significant

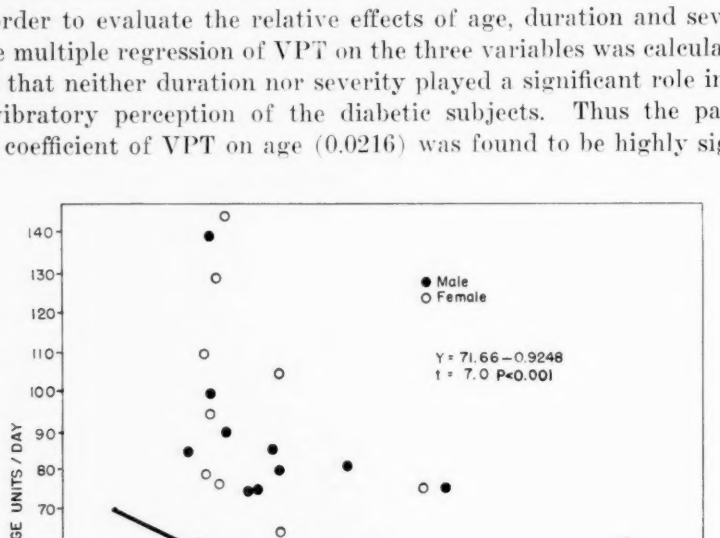

Fig. 6.—Relation between insulin requirements and age of patients.

whereas those of VPT on duration (0.0034) and on severity (0.0021) were insignificant. Further, the partial correlation coefficient of VPT on age (0.7288) was essentially the same as the multiple regression coefficient (0.7382), which indicates that neither duration nor severity play a significant role in determining the level of the VPT. Accordingly, the linear regression of the VPT on age is the only relation of importance in the group of patients with diabetes.

DISCUSSION

The analyses presented above reveal that the measurement of vibratory threshold must be expressed logarithmically rather than arithmetically; that there is a progressive impairment of vibratory perception with age; that the impairment is essentially similar on both sides of the body; that it is greater in the toes than in the fingers; that it is greater in the fingers and toes of patients with

diabetes than in those without this metabolic disorder; and that it is influenced significantly neither by the duration, nor the severity as gauged by the quantity of insulin required for regulation of the metabolic derangement.

It is well known that the acuity of vibratory perception declines with age in normal individuals. Likewise, it is known that the perception of vibration is symmetrical[16, 17] and that the acuity is less in the toes than in the fingers of healthy persons.[14, 17] The quantitative relationships between aging and impairment in vibratory perception, however, have received relatively little attention.[20-22] The data reported herein make it evident that comparison between groups of subjects cannot be made unless the influence of age is first isolated. Obviously, it would be erroneous to state that a group of subjects suffering from some specific syndrome has an impairment in vibratory perception unless consideration has been given to the ages of the members constituting the group and the comparison is made with healthy subjects of a similar age group. It is apparent also that the degree of impairment of vibratory perception cannot be evaluated unless comparisons are made between measurements at identical sites.

The precise nature of the mechanisms responsible for the impairment of vibratory acuity with aging or for the difference in the rates of impairment in the fingers and toes remains unknown. Of the various hypotheses which have been presented in explanation of these phenomena,[23] the most plausible is that which attributes the impairment in vibratory perception to a diminution in the blood supply to the peripheral nerves,[24-27] since arteriosclerosis is a frequent concomitant of aging and is more extensive in the lower than in the upper extremities.[28] In accord is Robert's demonstration[29] that the very abundant blood supply to the peripheral nerves may be obliterated by proliferative vasoplastic or thromboembolic processes involving the vasa nervorum and that the resultant ischemia may produce disturbances in the physiologic and morphologic integrity of these nerves.

The association of diabetes mellitus with increased impairment of vibratory perception may be accounted for by the fact that this metabolic derangement is associated also with an acceleration in the development of arteriosclerosis.[24-26] As Hart[30] has emphasized, "The changes in the arteries of the nondiabetic and diabetic are pathologically identical, the differences being that in the diabetic the changes come earlier." Likewise, the data reported herein reveal that the impairment in vibratory perception associated with the presence of diabetes comes on approximately twenty years earlier than in subjects without diabetes. Although it can be stated that premature development of arteriosclerosis and premature impairment of vibratory perception occur in the presence of diabetes, the nature of the definitive causal relationship is still to be established.

The demonstration that the impairment in vibratory acuity cannot be attributed to the duration of the clinical state or to the severity of the metabolic syndrome as gauged by the insulin requirements is in accord with the experience of many observers who have noted vascular disorders in patients with diabetes of the mildest degree.[31, 32] Not only is it generally acknowledged

that neither duration[33] nor severity of the diabetic state[32, 33] influence the development of arteriosclerosis but in many instances the vascular derangements occur even before the development of the classical symptoms of diabetes.[34] Accordingly, the development of arteriosclerosis and the impairment in vibratory perception must be regarded as concomitants rather than complications of the metabolic disorder.

The observations reported herein as well as the consensus in the literature indicate that the syndrome of diabetes mellitus in man is due to two independent components, one of which results in insulin insufficiency and the other in an accelerated rate of neurovascular damage. The factors responsible for the development of either one of the components are still unknown.[35]

SUMMARY AND CONCLUSIONS

A method for the quantitative measurement of the threshold of vibratory perception is described.

The threshold for the perception of vibratory stimuli increases with age. This increase is similar on both sides of the body, is greater in the toes than in the fingers, and is essentially the same in men and women.

The presence of diabetes results in a marked impairment of vibratory acuity, but the influence of age remains unchanged so that the patient with diabetes reacts as if he were approximately twenty years older in this respect than a person without diabetes.

The impairment of vibratory acuity in patients with diabetes is not dependent upon either the duration or the severity of the metabolic syndrome and consequently should be regarded as a concomitant rather than a "complication" of the metabolic derangement due to insulin insufficiency.

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BIOCHEMICAL CHANGES IN SERUM AND FECES DURING INGESTION OF CARBOXYLIC, SULFONIC, AND ANION EXCHANGE RESINS

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THE efficacy of various forms of synthetic cation exchangers or resins in augmenting fecal sodium and potassium has been described in previous communications from this laboratory.[1-6] These and other published reports agree that on a daily intake of the acidifying type exchangers, i.e., in the hydrogen or ammonium form, carboxylic or sulfonic type, one can in general anticipate up to 1.5 milliequivalent increments in univalent stool cations per gram of ingested resin.[7-10] The intake of such resins necessary for clinically significant deviations of electrolytes to stools may prove burdensome to some patients. In others, particularly in those with renal disorders, an undesirable degree of acidosis can develop. We have, therefore, tried combinations of synthetic exchangers, both the cationic and anionic types, in the hope of overcoming these two limitations.

MATERIALS AND METHODS

The nitrogen, chloride, sodium, and potassium content of stools, the bicarbonate, chloride, sodium, potassium, calcium, phosphorus, and protein concentrations in serum, and the nonprotein nitrogen and sugar levels of blood have been determined[3] in a total of 19 subjects (diabetes mellitus, 17; obesity, 1; cirrhosis of the liver, 1) without evident renal disease. They were maintained on one of the following resin combinations:

A) Carboxylic, H^+ form (50 per cent) + sulfonic, H^+ form (50 per cent)
Carboxylic, NH_4^+ form (50 per cent) + sulfonic, NH_4^+ form (50 per cent)

B) Carboxylic, H^+ form (50 per cent) + alkylene polyamine resin* (50 per cent)
Carboxylic, H^+ form (40 per cent) + alkylene polyamine resin (60 per cent)

C) Carboxylic, H^+ form (40 per cent) + carboxylic, K^+ form (10 per cent) + alkylene polyamine resin (50 per cent)
Carboxylic, H^+ form (59 per cent) + carboxylic, K^+ form (29 per cent) + alkylene polyamine resin(12 per cent)

The resin was administered in equal doses immediately after meals and at bedtime suspended in water with or without added cornstarch and sugar.

RESULTS

(A) *Effects of Mixtures of Acidifying Carboxylic and Sulfonic Resins.*—The ingestion of H^+ or NH_4^+ form carboxylic and sufonic resins, 30 or 40 Gm. of

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*Strong anion exchanger.

TABLE I. BODY WEIGHT AND ANALYSES OF BLOOD AND SERUM IN DIABETIC CHILDREN RECEIVING H^+ FORM CARBOXYLIC AND SULFONIC RESINS IN EQUAL AMOUNTS

PATIENT	TIME*	THERAPY		BODY WT.	BLOOD		SERUM					
(AGE-SEX)	(DAYS)	RCOOH (GM./D.)	RSO_3H (GM./D.)	(KG.)	SUGAR (MG. %)	NPN (MG. %)	HCO_3 (MEQ./L.)	CL (MEQ./L.)	NA (MEQ./L.)	K (MEQ./L.)	CA (MG. %)	P (MG. %)
M. J.	0	0	0	30.4	249	36	24.4	95.3	139	5.0	10.7	4.9
12 M	0- 5	0	0	30.2	321	28	25.6	91.0	143	5.2	10.5	4.6
	5- 7	40	40	30.2	303	28	15.7	106.5	141	4.6	–	4.3
	7-10	40	40	30.2	361	33	13.5	105.3	134	–	9.9	6.2
M. B.	0	0	0	33.8	254	43	22.9	94.8	138	4.7	11.0	4.6
11 F	0- 5	0	0	34.0	220	39	22.7	96.8	142	–	–	4.2
	5- 7	40	40	33.2	322	36	17.3	103.4	137	4.7	10.8	4.2
	7-10	40	40	33.6	174	35	20.9	97.3	130	4.1	–	4.8
K. R.	0	0	0	32.8	216	38	22.2	97.4	154	4.9	10.7	4.7
15 M	0- 5	0	0	33.6	129	36	24.4	95.2	149	4.9	10.1	5.3
	5- 7	40	40	33.8	352	37	15.7	106.5	138	4.6	10.3	5.6
	7-10	40	40	33.4	185	36	16.4	103.7	135	3.7	10.1	4.2
J. C.	0	0	0	54.4	355	38	20.1	94.4	138	4.6	10.2	4.2
15 F	0- 4	0	0	55.0	356	37	19.2	94.9	138	5.2	10.4	3.9
	4- 6	30	30	55.0	–	–	18.5	103.0	133	4.3	–	–
	6- 8	30	30	54.2	345	41	16.2	105.2	130	3.8	9.8	3.6
M. P.	0	0	0	52.0	264	28	25.5	95.6	138	4.2	9.8	4.3
15 F	0- 5	30	30	52.8	145	34	22.0	103.5	144	4.0	10.7	3.9
	5- 6	0	0	52.6	256	30	21.4	109.4	135	4.3	–	3.5
	6- 7	0	0	54.0	210	31	26.1	102.4		4.0	9.9	3.8
B. S.	0	0	0	32.2	110	36	23.7	94.8	141	5.0	10.4	4.5
12 F	0- 4	0	0	33.8	100	33	28.3	98.9	137	4.6	10.5	5.0
	4- 6	30†	30†	33.6	300	32	16.4	104.1	133	4.4	–	4.2
	6- 9	30†	30†	33.6	112	18	19.7	104.8	138	4.1	–	4.9

*Values obtained at end of indicated interval.
†$RCOONH_4$ and RSO_3NH_4.

TABLE II. INTAKE AND STOOL DATA IN DIABETIC CHILDREN RECEIVING H^+ FORM CARBOXYLIC AND SULFONIC RESINS IN EQUAL AMOUNTS

SUBJECT	TIME (DAYS)	THERAPY		INTAKE					STOOL EXCRETION								
									TOTAL					PER GRAM RESIN			
		RCOOH (GM./D.)	RSO_3H (GM./D.)	FLUID (L.)	CL (MEQ.)	NA (MEQ.)	K (MEQ.)	TN (GM.)	WT. (GM.)	CL (MEQ.)	NA (MEQ.)	K (MEQ.)	TN (GM.)	CL (MEQ.)	NA (MEQ.)	K (MEQ.)	TN (GM.)
M. J.	0- 5	0	0	14.8*	363	300	426	94.5	252	3	7	78	4.1				
	5-10	40	40	14.9*	306	257	366	81.1	274	5	11	168	3.2	0.01	0.03	0.42	0.01
M. B.	0- 5	0	0	15.9*	363	300	426	94.5	339	3	11	61	7.1				
	5-10	40	40	14.2*	363	300	426	94.5	319	2	67	177	2.5	0.01	0.17	0.44	0.01
K. R.	0- 5	0	0	18.8*	496	409	582	129.1	405	6	5	139	6.9				
	5-10	40	40	18.7*	472	389	554	122.8	230	2	117	190	3.7	0.01	0.29	0.48	0.01
J. C.	0- 4	0	0	7.6		Diabetic diet			111	1	3	16	1.6				
	4- 8	30	30	6.6		Diabetic diet			714	5	166	252	7.5	0.00	0.69	1.05	0.03
M. P.	0- 5	30	30	14.0*	299	247	352	77.9	110	–	10	91	1.6	–	0.03	0.30	0.01
B. S.	0- 4	0	0	7.6		Diabetic diet			212	0	4	27	4.2				
	4- 9	30†	30†	9.8		Diabetic diet			740	1	338	12	7.2	0.00	1.13	0.04	0.02

*Whole milk formula.
†$RCOONH_4$ and RSO_3NH_4.

TABLE III. BODY WEIGHT AND ANALYSES OF BLOOD AND SERUM IN SUBJECTS RECEIVING COMBINATIONS OF AN H^+ FORM CARBOXYLIC RESIN AND AN ALKYLENE POLYAMINE EXCHANGER

SUBJECT (AGE-SEX)	TIME* (DAYS)	THERAPY RCOOH (GM./DAY)	THERAPY A.P.E. (GM./DAY)	BODY WT. (KG.)	BLOOD SUGAR (MG. %)	BLOOD NPN (MG. %)	SERUM HCO_3 (MEQ./L.)	SERUM CL (MEQ./L.)	SERUM NA (MEQ./L.)	SERUM K (MEQ./L.)	SERUM CA (MG. %)	SERUM P (MG. %)	SERUM PROTEIN (GM. %)
R. D.	0	0	0	74.4	–	38	25.4	100.2	–	4.5	10.7	4.7	7.5
13 M	0- 4	0	0	74.8	96	34	26.1	101.8	139	4.8	–	4.5	7.5
Obesity	4- 8	40	40	74.8	85	35	25.7	95.9	138	4.1	10.2	4.6	7.4
J. J.	0	0	0	26.4	273	39	17.4	97.0	144	5.9	10.3	4.0	6.7
7 F	0- 5	0	0	26.4	353	40	16.0	96.0	134	5.3	–	3.9	7.0
Diabetes	5- 7	40	40	26.2	123	32	20.4	102.8	139	3.8	9.1	5.0	6.3
	7-11	40	40	26.2	359	39	16.7	97.4	135	4.3	–	3.7	6.5
B. B.	0	0	0	48.2	254	38	22.6	99.1	134	5.5	11.6	4.0	7.1
14 F	0- 4	0	0	49.4	161	32	24.8	103.4	138	4.8	10.2	4.1	6.9
Diabetes	4- 8	40	40	48.2	225	34	20.9	96.5	138	4.5	–	3.8	7.1
L. T.	0	0	0	31.0	151	38	25.3	99.1	137	5.5	10.5	4.4	–
9 M	0- 5	0	0	31.0	211	37	23.8	97.0	144	5.0	10.2	4.6	–
Diabetes	5- 7	40	40	30.4	326	42	19.1	100.6	132	5.1	9.6	4.2	–
H. D.	0	0	0	50.0	226	43	25.1	101.8	141	5.0	10.0	3.6	6.8
16 F	0- 5	0	0	49.0	251	45	22.5	101.3	138	4.1	10.1	4.1	–
Diabetes	5- 9	40	40	49.4	230	37	17.2	108.0	137	3.4	10.1	3.7	7.2
	9-12	40	40	49.2	183	37	17.3	111.3	136	3.7	9.8	3.8	6.7
W. S.	0	0	0	29.8	185	30	24.9	100.4	–	5.1	10.9	4.8	7.4
9 M	0- 5	0	0	30.0	169	31	25.2	97.2	138	4.9	10.6	4.7	7.2
Diabetes	5- 7	40	60	30.2	147	31	22.1	101.5	136	4.4	10.3	5.0	6.9
	7-10*	40	60	30.6	156	28	25.1	99.4	139	4.2	–	–	–

*Values obtained at end of indicated interval.

each per day for a total of four or five days was invariably accompanied by a rise in serum chloride and a decrease in serum CO_2 content (Table I). The serum potassium values fell in each patient, minimally in M. P., but in no instance did hypokalemia result. Sodium concentrations were definitely lowered in the serum of all of the subjects. Serum calcium and phosphorus, as well as blood nonprotein nitrogen and sugar values, fluctuated about pretreatment levels without discernible pattern. Glycosuria did not increase in these diabetic subjects, nor did ketonuria develop.

Prior to resin ingestion stool sodium excretion ranged between 0.8 and 2.2 milliequivalents per day; on resin, it ranged between 2.0 and 66.3 (0.03 to 1.1 milliequivalents per gram of ingested resins) (Table II). The highest two values were recorded in J. C. and B. S. while on a diabetic diet unlimited as to sodium content. The excretion of potassium exceeded that of sodium both in the control and in the treatment periods in all patients but one, B. S. We have no explanation for the low stool potassium during therapy in this child. With the exception of this one patient the resins produced larger increments in stool potassium than in stool sodium. During therapy the daily output of the former ion in stools ranged up to 63.0 milliequivalents. The minimum and maximum losses of sodium and of potassium together per gram of exchangers ingested were 0.3 and 1.7 milliequivalents, respectively. These values have not been corrected for the excretion of electrolytes which would have occurred if resins had not been given.

The exchangers did not produce any discernible alterations in stool chloride or nitrogen.

(B) *Studies with Cation-Anion Exchange Resin Combinations.*—The rises in chloride and concomitant decreases in serum bicarbonate seen with the acidifying resins were recorded less often during the ingestion of a combination of a carboxylic resin (H^+ form) and a strong anion exchanger (Table III). Decreases in serum potassium did occur just as frequently. No characteristic alterations were seen in any of the other constituents, though serum calcium fell 0.9 milligram per cent or more in J. J., B. B., and L. T., and the last of these developed a sodium level of 132 milliequivalents after two days of therapy.

The composition of stools did not clearly differ from that observed with the carboxylic-sulfonic resins, since the highest and lowest losses of sodium and potassium per gram of resins were 0.2 and 1.9 milliequivalents (Table IV) during maintenance on regular diabetic diets. In addition, when the data are calculated in terms of the cation exchanger alone, there is no consistent evidence of a higher efficiency. The one patient, H. D., who excreted 3.8 milliequivalents of sodium and potassium per gram of ingested carboxylic resin, further differs from the rest of the group in that the stool mass was so great.

(C) *Serum and Stool Composition During Ingestion of H^+ and K^+ Form Carboxylic Resins Together With Anion Exchanger.*—These combinations of resins differed from the two preceding in their effects on serum electrolytes. Serum potassium concentrations did not fall as far, and only in M. R. did the bicarbonate values decrease (Table V). There were no consistent changes in the other measured serum constituents. Univalent cations excreted in stools dur-

TABLE IV. INTAKE AND STOOL DATA IN SUBJECTS RECEIVING COMBINATIONS OF AN H^+ FORM CARBOXYLIC RESIN AND AN ALKYLENE POLYAMINE EXCHANGER

SUBJECT	TIME (DAYS)	THERAPY: RCOOH (GM./DAY)	THERAPY: A.P.E. (GM./DAY)	DIET	STOOL EXCRETION: TOTAL WT. (GM.)	TOTAL CL (MEQ.)	TOTAL NA (MEQ.)	TOTAL K (MEQ.)	TOTAL TN (GM.)	PER GRAM RESIN CL (MEQ.)	PER GRAM RESIN NA (MEQ.)	PER GRAM RESIN K (MEQ.)	PER GRAM RESIN TN (GM.)	PER GRAM RCOOH CL (MEQ.)	PER GRAM RCOOH NA (MEQ.)	PER GRAM RCOOH K (MEQ.)	PER GRAM RCOOH TN (GM.)
R. D.	0- 4	0	0	Full	519	2	6	50	5.6								
13 M	4- 8	40	40	Full	352	6	12	–	3.5	0.02	0.04	–	0.01	0.04	0.08	–	0.02
J. J.	0- 5	0	0	Diabetic	187	10	21	2	5.7								
7 F	5-11	40	40	Diabetic	211	5	75	36	3.6	0.01	0.16	0.08	0.01	0.02	0.32	0.16	0.02
B. B.	0- 4	0	0	Diabetic	–	–	–	–	–								
14 F	4- 8	40	40	Diabetic	584	5	26	128	13.0	0.00	0.08	0.40	0.04	0.00	0.16	0.80	0.08
L. T.	0- 5	0	0	Diabetic	337	4	6	44	3.9								
9 M	5- 7	40	40	Diabetic	301	6	3	55	3.1	0.04	0.02	0.34	0.02	0.08	0.04	0.68	0.04
H. D.	0- 5	0	0	Diabetic	409	2	5	65	7.0								
16 F	5-12	40	40	Diabetic	1805	32	473	587	42.8	0.06	0.84	1.05	0.08	0.11	1.68	2.10	0.15
W. S.	0- 5	0	0	Diabetic	512	6	16	73	7.0								
9 M	5-10	40	60	Diabetic	663	3	41	153	9.0	0.01	0.08	0.31	0.02	0.02	0.25	0.76	0.04

TABLE V. BODY WEIGHT AND ANALYSES OF BLOOD AND SERUM IN SUBJECTS RECEIVING H^+ AND K^+ FORMS OF THE CARBOXYLIC CATION EXCHANGER IN COMBINATION WITH AN ALKYLENE POLYAMINE RESIN

SUBJECT (AGE-SEX)	TIME* (DAYS)	THERAPY: RCOOH (GM./DAY)	THERAPY: RCOOK (GM./DAY)	THERAPY: A.P.E. (GM./DAY)	BODY WGT. (KG.)	BLOOD: SUGAR (MG. %)	BLOOD: NPN (MG. %)	SERUM: HCO_3 (MEQ./L.)	SERUM: CL (MEQ./L.)	SERUM: NA (MEQ./L.)	SERUM: K (MEQ./L.)	SERUM: CA (MG. %)	SERUM: P (MG. %)	SERUM: PROTEIN (GM. %)
M. R.	0	0	0	0	31.8	123	35	24.6	102.6	144	4.2	–	4.9	7.3
9 F	0- 2	24	6	30	31.6	105	31	19.2	103.6	141	4.0	10.4	4.6	6.8
Diabetes	2- 5	24	6	30	31.6	131	29	21.6	104.2	140	4.4	10.4	4.1	6.5
J. B.	0	0	0	0	63.6	217	37	25.0	98.7	144	3.8	10.6	3.8	6.8
15 M	0- 2	24	6	30	63.6	246	32	25.5	99.2	143	4.1	10.5	3.8	6.4
Diabetes	2- 5	24	6	30	64.2	242	34	24.6	99.9	144	4.3	10.6	3.1	6.8
N. K.	0	0	0	0	28.6	289	36	21.1	100.0	137	5.0	10.6	4.2	–
10 F	0- 2	32	8	40	28.6	277	37	22.4	100.2	133	4.4	–	4.1	–
Diabetes	2- 5	32	8	40	29.0	–	36	20.6	103.1	146	5.2	9.9	4.1	–
R. B.	0	0	0	0	–	101	31	16.0	96.3	129	5.6	8.5	–	5.7
46 M	0- 5	35.4	17.4	7.2	–	95	29	21.4	96.8	130	5.5	8.5	3.9	–
Cirrhosis	5-10	0	0	0	–	90	27	19.2	98.6	133	5.2	–	4.0	5.6
R. S.	0	0	0	0	54.0	264	31	24.2	96.5	143	4.7	9.4	4.0	6.9
14 M	0- 2	23.6	11.6	4.8	54.6	245	29	24.5	98.3	132	4.3	9.8	4.4	6.8
Diabetes	2- 5	23.6	11.6	4.8	54.2	220	30	25.0	102.6	148	5.0	9.0	–	6.8
W. I.	0	0	0	0	32.2	255	40	25.8	91.4	142	5.6	–	4.3	7.4
11 M	0- 2	35.4	17.4	7.2	32.0	273	36	–	98.7	141	4.7	10.1	4.3	7.2
Diabetes	2- 5	35.4	17.4	7.2	32.6	229	31	28.3	97.9	150	5.3	10.5	4.4	6.5
E. O.	0	0	0	0	46.2	275	31	21.4	100.2	142	4.6	–	–	7.1
15 F	0- 2	35.4	17.4	7.2	46.2	268	30	20.4	96.6	140	5.3	11.2	4.3	6.8
Diabetes	2- 5	35.4	17.4	7.2	46.8	243	30	23.6	101.6	138	4.3	10.3	4.1	7.2

*Values obtained at end of indicated interval.

TABLE VI. INTAKE AND STOOL DATA IN SUBJECTS RECEIVING H^+ AND K^+ FORMS OF THE CARBOXYLIC CATION EXCHANGER IN COMBINATION WITH AN ALKYLENE POLYAMINE RESIN*

SUBJECT	TIME (DAYS)	THERAPY: RCOOH (GM./DAY)	THERAPY: RCOOK (GM./DAY)	THERAPY: A.P.E.* (GM./DAY)	DIET	STOOL EXCRETION: TOTAL WT. (GM.)	TOTAL CL (MEQ.)	TOTAL NA (MEQ.)	TOTAL K (MEQ.)	TOTAL TN (GM.)	PER GRAM RESIN CL (MEQ.)	PER GRAM RESIN NA (MEQ.)	PER GRAM RESIN K (MEQ.)	PER GRAM RESIN TN (GM.)	PER GRAM CARB. RESIN CL (MEQ.)	PER GRAM CARB. RESIN NA (MEQ.)	PER GRAM CARB. RESIN K† (MEQ.)	PER GRAM CARB. RESIN TN (GM.)
M. R. 9 F	0- 5	24	6	30	Diabetic	701	8	124	154	17.9	0.03	0.41	0.51	0.06	0.06	0.82	1.02	0.12
J. B. 15 M	0- 5	24	6	30	Diabetic	707	9	74	207	14.0	0.03	0.25	0.69	0.05	0.06	0.50	1.38	0.09
N. K. 10 F	0- 5	32	8	40	Diabetic	694	10	173	88	8.5	0.02	0.43	0.22	0.02	0.05	0.86	0.44	0.04
R. B. 46 M	0- 5	35.4	17.4	7.2	0.3 Gm. Na	974	4	9	278	9.6	0.01	0.03	0.93	0.03	0.02	0.03	1.05	0.04
	5-10	0	0	0	0.3 Gm. Na	1013	10	7	136	10.1								
R. S. 14 M	0- 5	23.6	11.6	4.8	Diabetic	–	–	–	–	–	–	–	–	–	–	–	–	–
W. I. 11 M	0- 5	35.4	17.4	7.2	Diabetic	894	9	180	290	10.4	0.03	0.60	1.00	0.04	0.00	0.68	1.10	0.04
E. O. 15 F	0- 5	35.4	17.4	7.2	Diabetic	514	6	204	218	7.2	0.00	0.68	0.73	0.02	0.02	0.77	0.82	0.03

*Alkylene polyamine resin (anion exchanger).
†Not corrected for K^+ originally bound to resin.

ing resin therapy ranged from 0.6 to 1.6 milliequivalents per gram (Table VI). Sodium excretion per gram of administered carboxylic resin tended to be slightly greater with this mixture than with the carboxylic-sulfonic or the carboxylic-anion exchangers described earlier (0.61 milliequivalent per gram vs. 0.39 and 0.42, respectively). Potassium excretion was also increased during ingestion of the hydrogen and potassium form carboxylic resins together with the anion exchanger, but this merely reflects the potassium supplied by the resin. Thus when six or eight grams of the potassium form of the carboxylic resin were administered each day, stool potassium was greater than or equal to the amount supplied by the exchanger (3.75 milliequivalents per gram of the potassium form); when larger amounts were ingested stool potassium was less than that present on the administered resin, indicating partial absorption from the intestinal tract.

DISCUSSION

The data clearly indicate that the administration of carboxylic and sulfonic resins together or of the carboxylic type with a strong anion exchanger did augment the excretions of sodium and potassium via the intestinal tract, but that this increment is only slightly greater, if at all, than that obtained when the carboxylic or sulfonic type is used alone. The hope that sulfonic exchangers would abstract cations at the pH of gastric juice while the carboxylic type would collect a further increment at the higher pH values of the bowel and thereby markedly raise fecal losses has not been realized. Neither have we been able to show any striking difference between the outputs of stool chloride, sodium, and potassium on cation-anion mixtures and that observed on cation exchangers alone. This is in keeping with the small changes reported in studies from other laboratories.[11-13] The failure of such combinations of cationic-anionic resins to augment stool electrolyte excretion to any marked or consistent degree beyond values seen with cation exchangers alone should be emphasized, since it indicates that this approach to raising resin efficiency is not promising. Although stool sodium was increased by an average of approximately 30 per cent, this increment in sodium is only 0.2 milliequivalent per gram of administered carboxylic resin in these experiments, and cannot be considered a clinically significant rise in efficiency. This is all the more valid when it is emphasized that the total bulk of administered resins is not thereby reduced.

Ingestion of any of these resin combinations may still be accompanied by significant though perhaps less marked alterations in acid-base and mineral metabolism, irrespective of the combination employed. There are at least two possible reasons to explain the lower incidence of hyperchloremia and acidosis recorded in these and other studies with anion-cation mixtures: (a) less of the cation exchanger was ingested, and (b) the anion resin, as suggested by Martz and associates, increased the excretion of anions in the feces, thereby decreasing the anion excretion load of the kidneys.[12] Neither of these two explanations is adequate, however, since sufficient acidifying resin (30 Gm. or more per day) was ingested to produce acidosis, and it is uncertain that the acidosis is merely a result of chloride retention under these circumstances. The chloride

retention may have been secondary to the acidosis. Irrespective of the explanation it is obvious that in renal insufficiency such a mixture would be preferable to acidifying carboxylic or sulfonic resins, were it not for the potassium retention which occurs with the use of a K^+ form resin.[4] Potassium intoxication is, however, an ever-present possibility in renal disease, per se, and hence K^+ form resins are usually contraindicated.[6, 14-16] It has also been pointed out that in most instances of clinical edema a measure of acidosis, akin to that produced by intermittent treatment with ammonium chloride, is desirable in treating sodium and water retention.[17-20]

The decreases in serum sodium concentrations to abnormally low values in all patients receiving the combination of sulfonic and carboxylic resins reflect the large amount of resin supplied each day (80 Gm.). Similar changes have occurred more slowly in patients and dogs receiving smaller amounts of carboxylic resin.[3, 36]

It seems reasonable to suggest that exchangers with higher efficiencies under biologic conditions are needed. This view in no wise ignores the demonstrated utility of the currently available agents in many clinical situations.[21-36]

SUMMARY

1. Equal amounts of acidifying carboxylic and sulfonic resins did not raise stool univalent cation output above that seen when a carboxylic exchanger was given in the same total dosage employed for the combination.

2. Combinations of cation-anion resins did not raise stool chloride, sodium, potassium, and nitrogen outputs consistently and markedly beyond levels observed with the same total dosage of cation exchanger given alone.

3. Admixture of an anion exchanger decreased the hyperchloremia and acidosis observed during the administration of an acidifying carboxylic exchanger.

4. The administration of more than eight grams per day of the potassium form of the carboxylic exchange resin was accompanied by partial absorption of the potassium into the body through the intestinal tract.

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THE ESTIMATION OF GAMMA-GLOBULINS IN NORMAL AND MYELOMATOUS PLASMAS BY CHEMICAL FRACTIONATION

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HYPERPROTEINEMIA is common in multiple myeloma, and fractionation studies by a variety of chemical and physical techniques have shown that abnormal plasma proteins may appear in the globulin fractions in many cases of that disease.[1] Numerous attempts have been made to relate the production of abnormal plasma proteins to the lesions of the disease and, inasmuch as the plasma cell is generally considered to be the characteristic cell of the myelomatous lesion, this cell has been implicated as a source of abnormal globulins.[2-4]

More recently, evidence has been accumulated to suggest that the plasma cell may be a site of antibody formation,[5-8] and plasmacytosis has been associated with diverse hyperglobulinemic states.[9] The constant association of hyperglobulinemia with the occurrence of plasma cells has not always been confirmed,[10] and the precise relationship of plasma cells to synthesis of serum globulins is not clear.[11] One of the reasons advanced to support the hypothesis that plasma cells synthesize antibody has been the association of hyperglobulinemia with multiple myeloma.[7, 8, 12] However, antibodies are generally gamma-globulins[13] and, although the abnormal plasma proteins that occur in multiple myeloma may at times have properties similar to those of gamma-globulins, in many cases the abnormal new proteins are distinctly separable from the gamma-globulins.[1, 4, 14, 15]

The data obtained by the use of salting-out procedures,[1] electrophoresis,[1, 4, 14, 15] or ultracentrifugation[1, 15, 16, 17] indicated that there is no single characteristic pattern of plasma proteins in myeloma, and suggested that further exploration of this problem should be performed using different methods for the separation of protein fractions. Further study of the relationship of gamma-globulins to the abnormal proteins found in multiple myeloma was, therefore, undertaken.

Cohn and his co-workers[18] have reported a fractionation method which permits the isolation of almost pure gamma-globulin. The adaptation of this method to small volumes of blood appeared desirable for clinical purposes and was attempted. While such an adaptation was being worked out, the studies of Lever and associates[19] appeared, indicating that the method of Cohn and co-workers could be applied successfully to the study of volumes of normal plasma as small as five milliliters. The method evolved by Lever and collabora-

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tors entailed the use of a bath refrigerated at –5° C., and filtration of the precipitates in the cold through sintered glass filters.

In the present study, the method of Cohn and associates was adapted to the separation of gamma-globulin from other plasma proteins. In the course of this separation, two other fractions were obtained, one containing albumin as its principal, but not sole component, and the other consisting primarily of globulins other than gamma-globulin. The method was applied to a study of blood obtained from nine patients with multiple myeloma and compared, in certain instances, with electrophoretic data.

MATERIALS AND METHODS

As published, Method 10 of Cohn and co-workers[18] requires the use of large quantities of plasma as starting material in order to extract those plasma proteins that are present in low concentrations. Their procedure was adapted to permit the isolation and extraction of the gamma-globulin fraction using small volumes of plasma or serum.

Apparatus.—In order to maintain the temperature at –5° C. during manipulations of the reagents, a cold bath composed of a mixture of chopped ice and calcium chloride was used. A metal container 10 inches by 15 inches by 10 inches was half filled with chopped ice, and a cupful of calcium chloride was added to bring the temperature down to –5° C. The temperature was readily and inexpensively maintained at –5.0° C. ± 0.5° C. by the occasional addition of more calcium chloride, and removal of excess water.

A portable refrigerated centrifuge (Model PR-1, International Equipment Company, Boston) was used throughout. When 2 ml. samples were to be fractionated, standard tapered 15 ml. centrifuge tubes were used. With larger samples, special heavy-walled, tapered tubes, measuring 150 mm. by 16 mm., were employed.*

TABLE I. EFFECT OF ACD ON GAMMA-GLOBULIN EXTRACTION

PLASMA ML.	ACD ML.	GAMMA-GLOBULIN (GM./100 ML. PLASMA)
5.0	0.0	0.59
4.0	1.0	0.55
3.5	1.5	0.53
2.5	2.5	0.52

Collection of Blood.—Blood was obtained by venepuncture, and coagulation was inhibited by the use of one drop of 20 per cent potassium oxalate solution (dried before use) for each four milliliters of blood. The plasma was separated as quickly as possible by centrifugation, and stored at –20° C. until needed. In several instances plasmas or sera were obtained from physicians who had stored them for varying periods of time. Inasmuch as the original conditions for the separation of the protein components of serum encompassed the use of acid-citrate-dextrose† (ACD) solutions, preliminary experiments were performed to determine the effect of the use of ACD on the separation of gamma-globulin from plasma. As is seen in Table I, the use of increasing amounts of ACD did not greatly influence the recovery of gamma-globulin from plasma. The use of serum instead of plasma from the same donor yielded similar results, and it was concluded that the variations in gamma-globulin values that were induced by the use of oxalated plasma or serum instead of ACD-treated plasma were within the limits of error of the method itself. It should be emphasized that the use of oxalated plasma or serum is justified only for the separation of gamma-globulins. The applicability of oxalated plasma to the separation of other fractions was not explored, but there is reason

*Obtained from Macalaster Bicknell Company, Cambridge, Mass.

†Acid citrate dextrose solution (ACD) contained 26.7 Gm. of trisodium citrate ($5\frac{1}{2}H_2O$), 8.0 Gm. citric acid monohydrate, and 22 Gm. of dextrose per liter of solution.

to anticipate that the separation of certain other plasma proteins would be affected by this modification of the procedure of Cohn and associates.

Reagents.—Detailed directions for making the various reagents used by Cohn and collaborators may be found elsewhere.[18, 19] The composition of the reagents used for the two extractions in the present study is as follows:

Reagent A, used for the initial separation, contained 250 ml. of 95 per cent ethanol, 2.4 ml. of sodium acetate buffer, and water to make one liter. The acetate buffer consisted of 2 ml. of 4M sodium acetate, 4 ml. of 10M acetic acid, and water to make 10 ml. This stock buffer was then diluted eighty times with water.

Reagent A', used to wash the initial precipitate, contained 10 ml. of 4M sodium acetate, 35 ml. of 1M acetic acid, 200 ml. of 95 per cent ethanol, and water to make one liter.

Reagent B, used to extract the gamma-globulins from the precipitate, consisted of 150 ml. of 95 per cent ethanol, 45 Gm. of glycine, 2 ml. of 1M sodium acetate, 1.4 ml. of acetic acid, and water to make one liter.

Reagent B', used to extract additional gamma-globulins from the precipitate, contained 150 ml. of 95 per cent ethanol, 45 Gm. of glycine, 5 ml. of 1M sodium acetate, 1.84 ml. of 1M acetic acid, and water to make one liter.

Aliquots of these reagents were cooled to −5° C. before addition to the protein solutions.

Procedure.—Step 1: Five milliliters of plasma in a 50 ml. Erlenmeyer flask was cooled to about 0° C. in the cold bath and 20 ml. of reagent A was added. A few milliliters of reagent A was initially allowed to run rapidly into the flask from a burette, and then the flow was adjusted so that 2 ml. per minute ran into the flask, which was continuously agitated. The flask was stoppered and left in the −5° C. bath for fifteen minutes.

Step 2: The contents of the flask were poured as completely as possible into a chilled 25 ml. centrifuge tube and the flask returned to the cold bath. The centrifuge tubes were balanced quickly, using an alcohol-water mixture, and centrifugated for forty-five minutes at 2,500 r.p.m. at −5° C. One or one and one-half hours were required for some samples. The clear, yellow supernatant was decanted into a test tube for subsequent analysis as supernatant "a." The centrifuge tube containing the precipitate was immediately returned to the cold bath.

Step 3: Ten milliliters of reagent A' was pipetted into the flask used for the initial separation (which had been kept in the cold bath) and the contents were rinsed into the centrifuge tube containing the precipitate. An additional 10 ml. of reagent A' was then added directly to the centrifuge tube. The precipitate was resuspended in the solution with a glass stirring rod and left in the cold bath for ten minutes.

Step 4: The tubes were then centrifugated at 2,800 r.p.m. for fifteen minutes at −5° C. and the supernatant wash was decanted and discarded. The centrifuge tube was immediately replaced in the cold bath.

Step 5: Ten milliliters of reagent B were pipetted into the centrifuge tube. With the aid of a glass stirring rod, the precipitate was broken up into small particles and left in the cold bath for one hour with occasional stirring.

Step 6: The tubes were then centrifugated at 2,500 r.p.m. for fifteen minutes at −5° C., and the clear, colorless supernatant was decanted into a test tube.

Step 7: Ten milliliters of reagent B' was pipetted into the centrifuge tube and the precipitate was resuspended with a glass stirring rod and left in the cold bath for ten minutes.

Step 8: The tubes were centrifugated at 2,800 r.p.m. for thirty minutes at −5° C. and the supernatant was decanted into the same test tube containing the supernatant derived from the addition of reagent B (Step 6) to form a combined supernatant "b" gamma-globulin fraction. The remaining precipitate was discarded.

When two milliliters of plasma were used as the starting material, a 25 ml. flask was used for the initial precipitation, and 15 ml. centrifuge tubes for the subsequent separations. The procedure was the same as that described above for five milliliters of plasma with the

TABLE II. PRECISION OF THE METHOD FOR SEPARATING GAMMA-GLOBULIN FROM POOLED NORMAL PLASMA

VOLUME OF PLASMA SAMPLE	
5 ML.	2 ML.
GAMMA-GLOBULIN (GM./100 ML.)	GAMMA-GLOBULIN (GM./100 ML.)
0.54	0.67
0.60	0.60
0.69	0.64
0.71	0.58
0.70	0.59
0.63	0.59
0.61	
0.58	
0.51	
0.53	
0.62	
0.57	
Mean 0.61	0.61
Standard deviation 0.068	0.036

following modifications of the amounts of the reagent: *Step 1:* 8 ml. reagent A; *Step 3:* 10 ml. reagent A′; *Step 5:* 5 ml. reagent B; and *Step 7:* 5 ml. reagent B′.

Chemical Determinations.—The total protein content of the plasma was determined by micro-Kjeldahl analysis and the protein content of supernatants "a" and "b" was determined by an application of the Biuret reaction[20] which was modified by Lever and associates.[19]

When five milliliters of plasma were used as starting material, the Biuret tests were performed on duplicate five milliliter aliquots of supernatant "b"; when two milliliters of plasma was used, duplicate four milliliter samples of supernatant "b" were analyzed. The inclusion of a reagent B′ blank tube to correct for the color due to glycine-copper complexes in the tubes containing the gamma-globulin fraction is essential. Solutions of human albumin were used as standards. These contained about one milligram of protein per milliliter in 0.9 per cent saline, with standardization by the micro-Kjeldahl method. The same standard may be used for both supernatant "a" (predominately albumin) and supernatant "b" (containing over 90 per cent gamma-globulin).[19]

Precision of the Method.—Five milliliter aliquots of a pooled human plasma preparation were separated into supernatants "a" and "b" which were analyzed for their protein content by the Biuret method. The results of twelve sets of analyses of five milliliter aliquots of the same pooled plasma are shown in Table II. The results of six sets of analyses for gamma-globulin using two milliliter aliquots of the same plasma pool are also shown. It is seen that the observed standard deviation for the separation of gamma-globulin under these conditions was as large as 11.0 per cent, and that there was no loss of precision as a consequence of using only two milliliter samples of plasma.

In the analysis of supernatant "a," composed largely of albumin, the standard deviation was 2.94 per cent for twelve samples of the same plasma pool.

TABLE III. PROTEIN CONTENT OF PLASMA AND SUPERNATANTS "A" AND "B" IN 15 NORMAL INDIVIDUALS

	MEAN	STANDARD DEVIATION
Plasma (Gm./100 ml.)	6.76	0.50 (7.4%)
Sup't "a" (Gm./100 ml. plasma)	5.21	0.62 (11.7%)
(per cent total protein)	77.0	7.1 (9.2%)
Sup't "b" (Gm./100 ml. plasma)	0.61	0.18 (29.5%)
(per cent total protein)	9.3	2.8 (30.1%)

The electrophoretic pattern of the pooled plasma used for studying the precision of the method is shown in Fig. 1. The gamma-globulin concentration of the pooled normal plasma calculated from the electrophoretic data was 0.60 Gm. per 100 ml. of plasma, compared to the value of 0.61 found by the chemical method.

RESULTS

Normal Individuals.—The determinations of the total protein content of plasma, supernatants "a" and "b" in a series of 15 healthy laboratory workers and blood donors are summarized in Table III. The protein content of super-

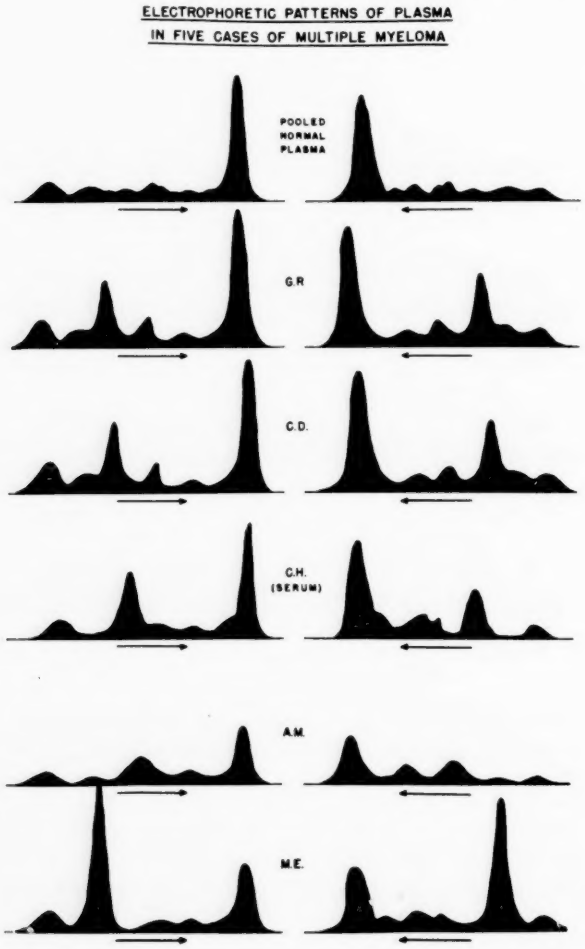

Fig. 1.

natant "a" in these normal individuals averaged about 10 mg. per milliliter. Since this fraction is a 1:5 dilution of plasma, this corresponded to 50 mg. per milliliter or 5.0 Gm. per 100 ml. of plasma and is composed primarily of albumin. Supernatant "b" averaged about 1.5 mg. per milliliter, corresponding to 6 mg. per milliliter or 0.5 Gm. per 100 ml. of plasma, and is composed of 90 to 100 per cent gamma-globulin.

The protein concentrations in these fractions are comparable to those obtained by the suction filtration method of Lever and associates.[19] The latter group reported that in normal individuals fractions IV + V + VI, corresponding to supernatant "a," averaged 67.8 per cent of the total plasma proteins, whereas fraction II, corresponding to supernatant "b," the gamma-globulin fraction, averaged 10.8 per cent of the total plasma proteins.

Multiple Myeloma Patients.—The conditions of separation that are the basis for the fractionation procedure used in this study have been designed for normal human plasma.[18] In the presence of normal plasma constituents, the gamma-globulins remain in the initial precipitate, probably in combination with lipoproteins. This protein-protein interaction is then diminished by increasing the dielectric constant of the medium with the dipolar ion, glycine. The gamma globulin is thereby released from the precipitate and is recovered in the supernatant.

TABLE IV. PARTIAL CHEMICAL FRACTIONATION OF PLASMA IN MULTIPLE MYELOMA

PATIENT	TOTAL PROTEIN (GM./100 ML.)	SUP'T "b" (GAMMA-GLOBULIN) (GM./100 ML. PLASMA)	SUP'T "b" (GAMMA-GLOBULIN) (% TOTAL PROTEIN)	SUPERNATANT "a" (GM./100 ML. PLASMA)	SUPERNATANT "a" (% TOTAL PROTEIN)
M. W.	8.3	4.0	48.0	2.74	33
N. K.	11.4	0.53	4.7	2.88	25
G. R.	5.1	0.31	6.1	4.31	85
A. M.	7.6	0.38	5.0	5.67	75
C. D.	7.35	0.31	4.2	4.47	65
C. H.*	7.45	0.20	2.7	6.12	82
J. B.	7.4	0.68	9.2	5.91	80
M. E.	8.4	1.83	22.0	3.30	39
E. O.	7.36	1.42	19.0	5.28	72
Normal†	6.8 ± 1.0	0.61 ± 0.36	9.3 ± 5.6	5.2 ± 1.2	77 ± 14

*Serum.
†Range of normal values from Table III. Mean ± 2 standard deviation.

Application of this method to the determination of gamma-globulin in plasmas of patients with disease must be made with considerable caution, inasmuch as the conditions for protein-protein interaction in pathologic plasmas may be different from those in normal plasma. Lever and associates[19] have shown that if the concentration of gamma-globulin is doubled by the addition of the pure protein to normal human plasma, the excess gamma-globulin is recovered in the proper fraction, indicating that this variation in the proportions of the proteins does not interfere with the system of fractionation. The effects on the accuracy of the method of wide variations in the amounts of other plasma proteins and of the presence of abnormal proteins, such as those in multiple myeloma, have not been previously reported. For this reason, electrophoretic analyses were obtained on some of the multiple myeloma plasmas and correlated with the chemical fractionation procedure.

Nine patients were included in the present study.* All have had the diagnosis of multiple myeloma proved by postmortem or biopsy demonstration

*We are grateful to Dr. Frank H. Gardner, Peter Bent Brigham Hospital, and Dr. William J. Harrington, First and Third Medical Services (Tufts), Boston City Hospital for plasma and serum from certain of these patients.

of typical multiple myeloma plasma cells in the bone marrow. The results obtained in this series of multiple myeloma patients using the chemical separation procedure described in this paper are shown in Table IV.

All the plasmas except that from patient G. R. had a high normal or elevated total plasma protein concentration. In three of the patients, M. W., A. E., and E. O., the gamma-globulin fraction was distinctly elevated. In the remaining six cases the gamma-globulin concentration was within the normal range or significantly below normal. Patient M. W., who had the highest gamma globulin value, was found at autopsy to have miliary tuberculosis in addition to multiple myeloma. Since the gamma globulin fraction may be elevated in miliary tuberculosis,[1] the high gamma-globulin concentration of 4.0 Gm. per 100 ml. found in this patient may not be entirely attributable to multiple myeloma.

Supernatant "a" represents only a rough measure of the albumin concentration; in none of the samples analyzed was the concentration of the albumin-containing fraction elevated, while in three of the samples the concentration of this fraction was markedly reduced, presumably due to hypoalbuminemia. In those patients with a high total protein and relatively low gamma-globulin concentration, the abnormal proteins remained in the final precipitate, as evidenced by the bulky residuum in the centrifuge tube after the gamma-globulins had been extracted. It was thus apparent that in many instances the abnormal globulins occurring in myeloma were not precipitated by this method in the gamma-globulin fraction. In most of the samples the gamma-globulin values were not elevated by this method, and in some they were distinctly depressed.

TABLE V. GAMMA-GLOBULIN VALUES OBTAINED BY CHEMICAL AND ELECTROPHORETIC FRACTIONATIONS

PATIENT	GAMMA-GLOBULIN (PER CENT OF TOTAL PROTEIN)	
	ELECTROPHORETIC	CHEMICAL
A. M.	8	5.0
C. H.	4	2.7
C. D.	10	4.2
G. R.	10	6.1
A. E.	53	22.0

Electrophoretic analyses of the plasma of four of these patients, and the serum of one were performed,* and the patterns are shown in Fig. 1. Abnormal components, accounting for about 25 per cent of the total area, appeared, and the mobilities of these components fell between those of the alpha$_2$ and gamma globulins in four cases. In patients C. H., C. D., and G. R., the mobility of the abnormal component was 1.8,† compared to the mobility of 1.0 for gamma-globulin. In patient A. M., the abnormal component migrated more rapidly, showing a mobility of 3.0. In all four of these patients the gamma-globulin peak was normal in contour but reduced in size. The plasma of the fifth patient, M. E., manifested an abnormal gamma-globulin peak, which was tall and thin in contour, and had a mobility of 1.1.

*We are indebted to Dr. John L. Oncley, of the University Laboratories of Physical Chemistry at the Harvard Medical School, for these analyses.

†All mobilities are expressed as $\times 10^{-5}$ cm.^{2}/volt/sec.

The comparison of the gamma globulin values obtained by chemical fractionation and by electrophoresis, in Table V, shows that although there is good qualitative agreement between the two methods, in that both methods demonstrated the high gamma-globulin levels in M. E., and the low values in C. H., the quantitative agreement was not precise. The chemical method, in each instance, yielded values lower than those obtained by electrophoresis.

DISCUSSION

The requisite conditions for the separation of gamma-globulin fractions by the methods of Cohn and co-workers can be maintained with ease by the use of mixtures of calcium chloride and ice. The modifications utilized in this study were directed toward the separation of gamma-globulins from samples of normal plasma or serum, but it may be anticipated that application of the methods of Cohn and collaborators to the separation of other plasma proteins can be effected by appropriate utilization of the original method.

The application of this general procedure to the study of pathologic sera, however, presents new and complex problems. Although the gamma-globulin values obtained after chemical fractionation of normal plasmas agreed well with electrophoretic data, those obtained from sera or plasmas from patients with multiple myeloma were consistently lower than the electrophoretic values. In part, the differences between the two methods may be a reflection of the limitations of the chemical method in the range of low gamma-globulin values. On the other hand, although sample M. E. had high gamma-globulin levels by both methods, the value obtained by electrophoresis was over twice as great as the value obtained by chemical fractionation. In this instance, the gamma-globulin content was so high that conceivably two routine extractions for separation of this protein were inadequate, and further extractions might have diminished the discrepancy. Unfortunately, further supplies of this patient's plasma were not available.

The discrepancy between the values for gamma-globulin obtained by chemical fractionation as compared with electrophoresis may also stem from indications that some of the abnormal proteins found in multiple myeloma interact with normal serum proteins,[21] and the altered properties of such proteins could conceivably cause them to behave quite differently when fractionated by the two methods.

The many studies of the chemical and physical properties of plasma proteins in multiple myeloma emphasize the lack of correlation between the relative concentrations of the different fractions as determined by chemical fractionation, electrophoresis, ultracentrifugation, and immunologic study, and indicate the complex and variable nature of the serum changes in multiple myeloma.[1, 4, 14, 15, 16, 17, 21, 22, 23] Abnormal components have been demonstrated by a variety of procedures to be globulins of varying properties which at times coincide in one or another measurement with properties of normal serum globulins, and at times may appear as entirely new fractions. This heterogeneity of serum proteins is in contrast to the usual patterns of response to infections or to hepatic disease, when there are alterations in the content of the normal serum globulins,

without evidence of abnormal proteins.[1] It has been suggested that the heterogeneity of the serum proteins in multiple myeloma may be a result of a deranged synthetic mechanism which would otherwise produce normal proteins, but there is as yet no direct evidence bearing on this possibility.[2-4]

The gamma-globulin values obtained by chemical fractionation in this study are consistent with the evidence that a variable serum protein pattern may occur in multiple myeloma. Only three of the nine samples studied manifested increased amounts of gamma-globulin, and the plasma with the highest concentration of gamma-globulin was obtained from a patient later found to have miliary tuberculosis in addition to multiple myeloma. Hyperglobulinemia was not necessarily accompanied by increased concentrations of gamma-globulins in the samples studied by chemical fractionation or by electrophoresis.

SUMMARY

The chemical method for the fractionation of plasma, as developed by Cohn and colleagues, has been applied to the determination of concentrations of gamma-globulin in small samples of plasma. By the use of calcium chloride-ice mixtures, the requisite conditions of temperature were maintained with ordinary laboratory equipment. Either plasma or serum may be used for the determination of gamma-globulin concentrations, and oxalate or acid-citrate-dextrose may be used as an anticoagulant. The precision of the determination of gamma-globulin is about 11.0 per cent, and the mean gamma-globulin value obtained from fifteen normal plasmas was 0.61 ± 0.18 Gm. per 100 ml. of plasma. Plasma or serum from nine patients with multiple myeloma gave gamma-globulin values within or below the normal range in six, and elevated values in three. The values by chemical fractionation were consistently lower than those obtained by electrophoresis, but the two methods were in good qualitative agreement. The findings support the indications that the hyperglobulinemia of multiple myeloma is not necessarily associated with increased concentrations of gamma-globulins.

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PLASMA AND ERYTHROCYTE SODIUM AND POTASSIUM CONCENTRATIONS IN A GROUP OF SOUTHERN WHITE AND NEGRO BLOOD DONORS

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BECAUSE of our interest in the metabolism of electrolytes in normal man and in patients with congestive heart failure, it was considered advisable to investigate further the turnover phenomena of sodium, potassium, and chloride between the cells and extracellular fluids of man. These studies were undertaken to elucidate other observations obtained with radioactive isotopes in man as a whole.[1, 2] Erythrocytes were selected for special investigation because they are readily separated from their surrounding plasma and because they are probably disturbed relatively little when observed in vitro. As an aid in the interpretation of isotope experiments, the concentration of sodium and potassium in erythrocytes and plasma was measured in one hundred five people. Subjects were obtained from those who offered donation of blood to the Blood Bank of the Charity Hospital. The influence of sex, age, race, fasting, and menstruation was evaluated in the observations, and the absolute concentrations of sodium and potassium in the plasma and erythrocytes of these subjects were recorded.

MATERIAL AND METHODS

Sixty-two male and forty-three female subjects, ranging in age from 18 to 60 years, constituted the series; forty-eight were white and fifty-seven were Negro subjects.

Their *state of health* was defined by the minimal requirements for donation of blood at Charity Hospital: temperature below 37.2° C., blood pressure between 200 and 110 mm. Hg. systolic and below 120 mm. Hg. diastolic, weight over one hundred ten pounds, and whole blood specific gravity in excess of 1.044. In thirteen subjects the blood pressure was higher than 160 mm. Hg. systolic, and in four it was higher than 95 mm. Hg. diastolic. The mean electrolyte concentrations in this group were not significantly different from the remainder of the series. The duration of fasting and the stage of the menstrual cycle in female subjects were recorded.

The *blood samples were collected* immediately after 500 ml. of blood had been obtained from each donor and the tourniquet cuff had been deflated. Seven to 8 ml. was withdrawn through the 15-gauge phlebotomy needle into a dry syringe, and 5 ml. of this blood was then allowed to run from the tip of the syringe into a tube containing 1 mg. of heparin without preservative in 0.05 ml. of 0.15M solution of sodium chloride. All specimens were obtained within a two-week period, during which the average environmental temperature was 17° C.

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For *centrifugation*, the blood was poured into plastic tubes with diameters of 0.65, 0.95, or 1.11 cm., and the cells were packed at 20,000 RCF (relative centrifugal force) in an angle centrifuge. Although maximal detectable packing occurred in five minutes, centrifugation was continued for ten minutes.

Solutions for analysis in the flame photometer were prepared by simple dilution of the plasma and red cells with distilled water. Plasma was diluted five hundred times for sodium and twenty times for potassium determinations. The red cells were diluted 67.7 fold (0.15 ml. R.B.C. into 10 ml. water) for both determinations. Red cells were separated from plasma by the use of a special clamp (Fig. 1). After centrifugation of the blood and removal of part of the supernatant plasma for analysis, the clamp was applied to the plastic tube at a point about 2 mm. below the lowest level reached by the buffy coat during angle centrifugation. The jaws of the clamp pushed the walls of the tube together to form a sealed lower

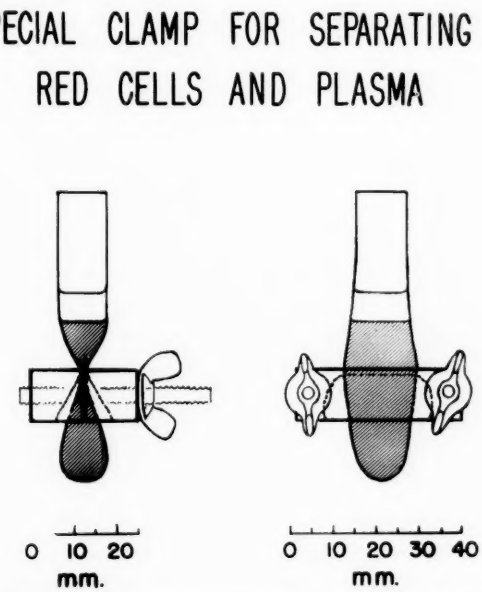

Fig. 1.—Diagram of the special clamp, drawn to scale, for isolating centrifuged red cells from the plasma. The clamp has been closed, isolating a column of packed R.B.C. at the bottom of the plastic tube from plasma, R.B.C., W.B.C., and platelets above. The upper part of the tube has not yet been removed.

chamber containing the bottom portion of the layer of red cells while simultaneously excluding the remaining supernatant plasma and over 95 per cent of the white cells (Fig. 1). This principle for obtaining little disturbed centrifugates from plastic tubes by sectioning the tube has been previously employed by Randolph and Ryan.[3] Because packed red cells are too viscous for accurate pipetting, the upper part of the tube was removed and discarded, and the clamped and sealed lower portion containing the erythrocytes for study was immersed in a mixture of dry ice and alcohol until the contents were frozen. After the hemolyzed red cells were thawed with running tap water, they could be pipetted readily.

Analyses for sodium and potassium were performed with a modified Beckman model DU flame photometer. A bracketing procedure, in which unknowns were compared with a higher and a lower standard in the same concentration range, was used.

EXPERIMENTAL ERROR

Because of differences in the values obtained by various investigators for content of sodium and potassium in plasma and erythrocytes, it is believed advisable to present certain

aspects of the studies concerned with an evaluation of the errors involved with various phases of the determinations.

1. *Chemical Determinations on Plasma.*—

(a) The *reproducibility* of results was studied in a series of determinations of sodium and potassium performed on aliquots of the same sample of plasma. For plasma sodium, the relative variability was 1.1 per cent, and for plasma potassium 1.5 per cent.

(b) *Recovery* of known amounts of sodium and potassium added to the diluted plasma averaged 95 per cent for each element.

(c) *Mutual excitation* caused by the high concentration of sodium in solutions of plasma analyzed for potassium produces falsely high values[4-6] unless a correction is made. To avoid such error, an equivalent amount of sodium (7 meq. per liter) was added to the standard solutions for determination of plasma potassium.

(d) *Injury of red cells by gravitational force* during high-speed centrifugation might allow potassium to escape into the plasma, producing falsely high values. However, in a series of duplicate samples of blood centrifuged at 20,000 RCF and at 750 RCF, no difference in the levels of plasma potassium was found.

2. *Chemical Determinations on Red Cells.*—

(a) The *reproducibility* of results in a series of analyses of aliquots of the same sample of red cells was: for sodium, a relative variability of less than 5 per cent, and for potassium, 1.6 per cent.

(b) *Recovery* of added sodium and potassium from a 67.7 fold aqueous dilution of red cells averaged 90 per cent for sodium and 95 per cent for potassium.

(c) *Error Due to Trapped Plasma:* Centrifuged red cells contain significant amounts of plasma trapped between them.[7-11] This factor is especially important in measurement of red cell sodium because the concentration of sodium in plasma is from ten to thirty-five times higher than in erythrocytes. Parpart and Ballentine[12] found that at centrifugal forces between 20,000 and 190,000 RCF the apparent hematocrit of centrifuged blood fell progressively and approached a value that was 1.7 per cent lower than the value obtained at 20,000 RCF. The difference is believed to be due to differences in degree of packing. The amount of plasma trapped between the red cells centrifuged and separated by the techniques used in this series was found to be 2.2 ± 0.04 per cent. This value was obtained by adding Na^{22} to whole blood and quickly separating and measuring the radioactivity of the plasma and red cells. Since it was known from other studies that a portion of the Na^{22} had entered the erythrocytes during centrifugation and separation, a value of 1.9 per cent was chosen for use in making corrections. This value was derived from data from other measurements of Na^{22} uptake of red cells to estimate the portion of the Na^{22} entering the red blood cells. Therefore, the results of all determinations on RBC were corrected for a plasma contamination of 1.9 per cent.

(d) *Error due to dilution of undigested R.B.C.:* The concentrations obtained for a simple aqueous dilution of the hemolyzed cells were compared with those for solutions of R.B.C. digested by the nitric acid and formic acid procedure used by Sheppard.[13] The values for red cell sodium were the same for both digested and undigested specimens, whereas the values for potassium were approximately 5 per cent higher for the digested R.B.C.

(e) *Influence of amount of dilution:* Dilutions of red cells higher than 67.7 fold showed no rise in apparent concentrations, such as might have been found if the lower dilution had a significantly higher viscosity than the standard solutions.

(f) *Error due to mutual excitation:* Because the concentration of potassium in the solutions of diluted red cells was ten times the sodium level, a correction for falsely high sodium values due to excitation of sodium by potassium was used. The correction factor was derived from the study of solutions containing known amounts of both sodium and potassium. For the average concentration of sodium in red cells, the corrections for plasma contamination

and for the high level of potassium were approximately equal in amount, and together they decreased the uncorrected value by approximately 5 meq. per liter.

(g) *Errors due to sodium contamination:* Since the sodium concentration of the solution of diluted red cells may be as low as 0.10 meq. per liter, strict precautions were employed to minimize sodium contamination from the distilled water and glassware.

(h) *Error due to size of tube:* The data were analyzed for any difference in the red cell sediments that could be correlated with the size of the tube used for centrifugation, but none was found.

(i) *Error due to contact of plasma and R.B.C.:* The continued contact of the plasma with the formed elements in the collected blood introduces errors due to net exchanges of sodium and potassium between the cells and plasma. The changes in red cell and plasma levels have been followed serially in one instance (Table I). The most noticeable changes were a fall in plasma potassium and red cell sodium due to a net uptake of potassium by

TABLE I. CHANGES IN CONCENTRATIONS OF SODIUM AND POTASSIUM OCCURRING IN BLOOD ALLOWED TO STAND VARYING LENGTHS OF TIME BEFORE CENTRIFUGATION.

TIME (MIN.)	CONCENTRATIONS MEQ./L.			
	PLASMA SODIUM	PLASMA POTASSIUM	RED CELL SODIUM	RED CELL POTASSIUM
6	143.5	3.8	6.1	77.2
30	144.5	3.6	6.0	77.8
51	143.5	3.5	5.7	77.9
82	143.5	3.4	5.5	79.9
111	144.0	3.5	5.7	81.2

the cells and a proportional loss in cellular sodium. With time, the directions of change are reversed, a well-known phenomenon.[13, 14] It occurs even when blood is maintained under simulated physiologic conditions. Because an average of twenty-nine minutes elapsed from the time the blood was withdrawn until it was centrifuged, the plasma potassium levels were averaged with a correction for the falsely low values resulting from delay in separation. This was done by plotting each plasma potassium value against the duration of standing for that sample and accepting as the true mean value that at the zero time intercept of the line of regression for this plot. By this correction, the mean was raised by 0.18 meq. per liter. This correction was omitted for the red cell sodium concentrations because its small magnitude was greatly outweighed by variations due to other factors.

RESULTS

Results are presented in detail in Table II, and the mean corrected values and other statistical data are included in Table III.

For each type of determination an estimate of the amount of variation in concentrations due to real differences between subjects was obtained by subtracting the error in reproducibility for that determination from the observed relative variability in the results for the entire series. This is considered the *biologic variation*. Inspection of Table III shows that the biologic variation was small for plasma sodium (1.2 per cent), but large for sodium within the red cells (22.5 per cent). The extent of intracellular and extracellular variation for potassium was similar (4.5 per cent and 7.6 per cent).

Scattergrams were made to determine the relationship of concentrations of sodium and potassium to the other data obtained on each donor. Apparently significant findings were subjected to statistical test (Student's test). Concen-

TABLE II. SUMMARY OF DATA ON ALL SUBJECTS

SPECIMEN NUMBER	SEX	RACE	AGE (YR.)	TIME* (MIN.)	LMP†	PLASMA SODIUM (MEQ./L.)	PLASMA POTASSIUM (MEQ./L.)	RED CELL SODIUM (MEQ./L.)	RED CELL POTASSIUM (MEQ./L.)
1	M	W	24	40	-	143.0	3.80	4.8	85.9
2	M	C	47	36	-	142.0	4.04	8.5	77.5
3	M	C	20	34	-	139.0	3.80	7.9	85.9
4	M	C	23	22	-	139.0	3.76	8.7	82.4
5	M	C	32	23	-	141.0	3.80	8.8	78.7
6	M	W	35	19	-	140.5	3.60	5.4	74.5
7	M	C	20	33	-	137.5	2.76	7.5	84.5
8	M	C	18	19	-	140.0	3.90	5.0	75.8
9	M	W	40	18	-	140.0	3.76	6.8	78.7
10	F	C	36	52	6 wk.	136.5	2.96	9.0	87.7
11	F	C	49	47	6 days	137.5	3.60	6.0	86.2
12	M	W	24	39	-	141.5	3.40	8.0	79.4
13	M	W	45	42	-	140.0	3.82	5.3	83.6
14	M	W	35	32	-	137.0	3.22	6.3	78.6
15	M	W	31	14	-	137.5	2.72	6.4	82.6
16	F	W	18	54	8 days	137.5	3.68	5.1	84.2
17	F	C	49	44	9 yr.	137.5	3.02	5.0	71.8
18	M	C	24	41	-	151.0	3.50	10.6	75.2
19	M	C	27	26	-	135.5	3.38	10.4	83.4
20	M	C	20	45	-	141.5	3.20	13.4	81.3
21	M	C	27	42	-	138.5	3.40	14.5	81.3
22	M	W	38	34	-	141.0	3.24	7.9	81.3
23	M	W	42	32	-	138.0	3.10	9.0	75.1
24	M	C	51	26	-	142.0	3.40	9.5	83.4
25	M	W	42	22	-	141.0	3.60	8.9	78.6
26	M	W	43	17	-	140.5	2.90	5.4	80.0
27	F	C	42	79	4 days	139.0	2.72	7.7	83.5
28	M	W	27	45	-	145.5	2.46	7.6	67.6
29	F	W	54	32	3 yr.	144.0	3.80	6.2	81.3
30	F	C	37	27	18 days	139.0	2.76	5.5	78.5
31	M	W	60	21	-	142.0	3.16	5.5	82.8
32	M	W	49	52	-	142.0	3.50	6.4	84.5
33	M	W	22	39	-	143.5	3.20	9.5	77.9
34	M	W	47	31	-	140.5	3.20	5.6	85.9
35	M	W	19	33	-	141.0	3.70	7.2	83.2
36	M	C	26	26	-	139.5	3.70	13.4	80.4
37	F	W	31	19	4 days	139.0	3.00	4.9	84.8
38	M	W	25	63	-	141.0	3.24	9.3	86.6
39	M	C	45	54	-	141.5	3.24	12.3	80.2
40	M	C	26	45	-	142.0	3.40	6.3	89.0
41	F	C	42	46	2 yr.	143.5	3.22	5.4	84.0
42	M	W	49	27	-	141.5	2.96	7.9	81.3
43	M	C	42	18	-	141.5	3.00	8.3	89.6
44	M	W	22	40	-	145.0	3.60	7.5	74.5
45	M	W	28	34	-	142.5	3.32	7.6	78.0
46	M	C	28	28	-	144.0	3.84	7.5	74.5
47	M	W	18	22	-	142.0	3.30	7.0	75.8
48	M	W	40	21	-	145.5	4.00	5.3	81.3
49	M	W	59	14	-	144.5	3.48	6.0	84.8
50	F	C	25	13	17 days	143.5	3.50	9.7	84.8
51	M	C	19	58	-	145.5	3.60	8.2	78.6
52	F	C	32	45	10 days	141.0	3.48	8.0	87.6
53	F	C	20	40	10 days	145.5	2.92	8.0	80.7
54	F	C	19	35	4 days	143.5	3.56	7.5	89.7
55	M	C	25	23	-	146.0	3.32	7.0	80.7
56	M	W	29	40	-	139.0	3.60	7.0	87.5
57	M	C	30	30	-	141.0	3.60	8.3	87.6
58	M	C	31	28	-	144.0	3.84	10.9	83.9
59	M	C	28	16	-	140.0	3.48	12.2	84.8
60	M	C	22	15	-	137.5	4.10	6.9	84.1

SPECIMEN NUMBER	SEX	RACE	AGE (YR.)	TIME* (MIN.)	LMP†	PLASMA SODIUM (MEQ./L.)	PLASMA POTASSIUM (MEQ./L.)	RED CELL SODIUM (MEQ./L.)	RED CELL POTASSIUM (MEQ./L.)
61	M	C	30	13	-	136.0	3.34	7.3	75.8
62	M	C	49	25	-	138.5	3.46	6.6	81.3
63	M	C	31	20	-	141.0	3.52	4.0	90.3
64	M	C	27	13	-	143.5	3.78	5.5	87.6
65	M	C	26	14	-	139.5	3.44	7.7	86.2
66	M	C	30	25	-	138.5	3.36	7.9	82.8
67	M	W	19	10	-	140.0	3.60	8.9	80.6
68	M	W	23	43	-	144.5	3.42	8.2	81.3
69	M	C	18	33	-	141.5	3.44	5.7	73.8
70	M	C	21	26	-	146.0	4.20	7.3	91.8
71	M	W	41	22	-	143.0	3.64	6.1	80.7
72	M	W	41	19	-	143.0	3.88	7.5	80.0
73	M	W	18	17	-	142.5	3.44	6.1	80.7
74	F	W	31	32	18 days	143.5	3.30	5.4	91.0
75	F	C	48	19	5 yr.	141.0	3.46	10.6	84.1
76	F	C	41	11	2 yr.	145.0	3.34	10.3	89.7
77	F	W	45	31	10 days	142.0	3.12	6.6	87.0
78	F	C	20	24	12 days	133.0	3.81	11.3	84.8
79	F	W	34	16	5 days	141.0	3.36	5.3	83.5
80	F	C	36	25	14 days	136.0	3.20	9.4	98.7
81	F	C	45	42	6 mo.	135.0	3.04	6.8	91.7
82	F	C	36	17	5 days	131.0	3.60	5.9	89.7
83	F	C	24	28	13 days	134.0	---	8.9	92.4
84	F	W	20	16	19 days	134.5	---	7.4	84.8
85	F	W	36	13	18 mo.	137.0	4.00	8.4	83.5
86	F	C	31	13	5 days	133.5	3.36	9.6	88.3
87	F	C	36	11	4 yr.	135.0	3.46	8.0	84.8
88	F	C	22	17	7 days	135.0	3.72	7.3	93.8
89	F	C	35	10	1 mo.	134.0	3.34	10.8	88.3
90	F	W	23	16	18 days	138.5	3.42	4.5	91.0
91	F	W	41	9	7 yr.	137.0	3.72	7.5	78.6
92	F	W	53	49	16 yr.	145.5	2.66	7.1	84.1
93	F	W	55	41	8 yr.	143.0	3.72	7.8	86.2
94	F	W	59	44	9 yr.	143.5	3.70	7.2	89.0
95	F	C	26	33	19 days	140.5	3.60	8.5	87.0
96	F	C	36	25	16 days	139.0	3.62	8.5	84.1
97	F	W	38	13	2 mo.	140.0	3.62	6.5	94.1
98	F	W	40	12	21 days	144.5	3.20	4.9	88.3
99	F	C	47	14	3 yr.	144.0	3.32	5.1	89.0
100	M	C	49	11	-	140.5	3.46	7.4	84.8
101	F	W	35	36	20 days	139.0	3.24	4.6	83.5
102	F	W	44	34	13 yr.	141.0	3.36	7.1	80.7
103	F	W	23	16	15 days	138.5	3.02	6.4	86.2
104	M	C	30	20	-	139.0	4.32	13.0	84.1
105	F	C	57	13	14 days	137.0	3.68	10.1	86.2

*Time refers to the duration of standing of the whole blood before separation of red cells and plasma.

†Last menstrual period.

TABLE III. COMPOSITE RESULTS OF ALL DETERMINATIONS

DETERMINATION	CONCENTRATIONS		RELATIVE VARIABILITY (PER CENT)	REPRODUCIBILITY ERROR (PER CENT)	BIOLOGIC VARIATION (PER CENT)	BIOLOGIC VARIATION (MEQ.)
	MEAN MEQ./L.	RANGE MEQ./L.				
Plasma sodium	140.6	131.0-150.5	2.3	1.1	1.2	1.7
Plasma potassium	3.62	2.45-4.30	9.1	1.5	7.6	0.24
Red cell sodium	7.7	4.0-14.5	27.5	5.0	22.5	1.7
Red cell potassium	83.4	67.6-98.5	6.1	1.6	4.5	3.8

tration of sodium and potassium in plasma and R.B.C. was not related to *age* (18 to 60 years) in this group. Likewise, concentrations of sodium and potassium in plasma and R.B.C. in the female subjects were not found to be correlated with the stage of the *menstrual cycle*. Although most of the donors were in an essentially *fasting state* when the blood was obtained, the concentrations of sodium and potassium in plasma and R.B.C. of subjects who had eaten during the previous four hours showed no differences from those who fasted. Each of the four concentrations measured was plotted against each of the others in the six possible combinations to search for a relationship between the level of *one cation and another*. No relationship was found.

The significance of differences associated with the *sex of the donor* may be decreased by the grouping of female subjects toward the end of the series. This resulted from a difficulty in obtaining female donors. The mean plasma sodium was 139.5 meq. per liter in the female subjects as compared to 141.3 meq. per liter in the male ($p = 0.006$); on the other hand, the red-cell potassium was higher in the female subjects, being 86.5 meq. per liter, as against 81.5 meq. per liter for the male ($p = 0.00001$).

Significant differences related to race were limited to the red cell sodium. In both male and female subjects, the red cell sodium was higher in Negroes. The mean values were: for the white male 7.0 meq. per liter, Negro male 8.7 meq. per liter ($0.001 < p < 0.01$), the white female 6.3 meq. per liter, and Negro female 8.1 meq. per liter (p 0.001). Racial differences become more evident when only the values over 9 meq. per liter are considered. Of the total of twenty donors in this group, seventeen were Negroes, and only three were white. All of the six red cell specimens containing over 12 meq. per liter were from Negro male subjects. After this racial difference for R.B.C. sodium was observed, the data were reviewed to learn if blood pressure, weight, or presence of syphilis might be correlated with red cell sodium. The blood pressures of the white and Negro subjects were not different. Although the Negro donors averaged fifteen pounds lighter in weight than the white donors, body weight and red cell sodium were not found to be related. Serologic test for syphilis, likewise, was not found to be related to the R.B.C. sodium.

Since measurements were made on each person at one instant only, determinations were made on one member of the laboratory staff nine times over a period of six months (Table IV—*A*). Considerable variation was recorded. In Table IV—*B* and IV—*C* the results of serial measurements on two Negro female patients with high concentrations of red cell sodium are shown to demonstrate that the level may remain high over three- to ten-day periods of observation.

DISCUSSION

A comparison of this series of plasma sodium and potassium determinations with those found in the literature reveals good agreement for levels of concentration of plasma sodium. Our potassium levels, however, are 10 per cent lower than the lowest reported by others.[15-17] Whether this is due to differences in technique or to actual variation of the donors is not known. Elliot and Holley[17] have analyzed a group of four hundred determinations and

found no differences associated with the sex of the donor, such as existed for the plasma sodium in this series. In the case of red cells, variations in the manner in which the cells and plasma were separated are probably largely responsible for the greater differences between values obtained,[6, 18-22] especially in the case of sodium. Our results are not inconsistent with the lowest values

TABLE IV. RESULTS OF DETERMINATIONS ON THE SAME INDIVIDUAL ON SEVERAL DIFFERENT OCCASIONS. *A* IS A NORMAL INDIVIDUAL, WHILE *B* AND *C* ARE HOSPITALIZED PATIENTS

DATE	CONCENTRATIONS (MEQ./L.)			
	PLASMA SODIUM	PLASMA POTASSIUM	RED CELL SODIUM	RED CELL POTASSIUM
A—Normal subject				
6/ 5/51	—	—	6.2	88.2
6/18/51	146.5	3.7	6.1	90.7
6/20/51	141.5	3.5	8.0	98.0
8/ 8/51	143.5	3.7	5.4	89.0
9/ 5/51	144.0	3.5	7.9	83.4
9/ 7/51	138.5	4.4	7.8	83.2
9/13/51	141.0	3.4	8.1	83.7
11/29/51	139.5	—	5.5	84.0
12/ 4/51	143.5	3.8	6.0	77.5
B—Hospitalized patient				
11/15/51	134.5	3.6	13.6	77.1
11/16/51	136.5	3.0	14.2	73.4
11/17/51	133.5	3.4	14.3	82.5
11/26/51	132.5	3.4	13.1	79.8
C—Hospitalized patient				
11/26/51	137.5	4.2	16.3	75.1
11/27/51	134.0	4.1	14.8	81.0
11/29/51	142.5	3.8	12.2	82.0

previously reported. The large range of red cell sodium concentrations found in this group of apparently healthy donors emphasizes the need for caution in attributing unusually high or low values to the effects of disease.

The higher concentrations of red cell sodium in Negroes may be related to the inclusion of persons with sickle-cell disease and sickle-cell trait in this group. If the cells of these donors sickled during study, enough extra plasma might be trapped between the red cells during centrifugation to produce an apparently significant difference between Negro and white donors on this basis alone. This explanation seems unlikely because: (1) studies with Na^{22} in two Negro patients with high red cell sodium showed the "red cell sodium" to be actually within the cells, (2) a statistical comparison of the red cell sodium of white and Negro donors using only that half of subjects of each group with the lowest values has shown the same significant differences that were found between the two groups as a whole. This would not be true if the only difference were due to spuriously high values in a minority of the Negro donors. The possibility remains, however, that factors other than race are responsible for the observed result.

SUMMARY

In a study of one hundred five blood donors, the mean concentration of sodium in the plasma was found to be 140.6 meq. per liter, and the concentration of potassium 3.62 meq. per liter.

A method has been described for obtaining the red cells from whole blood with a small and relatively constant amount of plasma contamination.

By use of this technique the mean red cell sodium was 7.7 meq. per liter of red cells and the potassium 83.4 meq. per liter.

The extent of biologic variation in these donors was analyzed and found to be small for plasma sodium but large for red cell sodium.

Differences in red cell sodium and potassium were related to some extent with the sex and race of the donor. Plasma sodium was lower and red cell potassium higher in female subjects. In Negroes the red cell sodium was definitely higher than in the white donors.

These differences have not been established to be due to the sex, race, or genetic constitution of the donor.

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THE PERMEABILITY OF THE DOUGLAS-TYPE BAG TO RESPIRATORY GASES

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DOUGLAS bags are widely used in cardiopulmonary studies for the collection of respiratory gases, and it is a prime requisite that they be impermeable to these gases. In his original paper in 1911, C. G. Douglas[1] stated that "the gas bag must be examined to see that the rate of diffusion of gases through its walls is negligible." The soundness of this advice recently became evident when low respiratory quotients were obtained from the analysis of expired air after it had remained in the Douglas bag for a period of time. A study was then undertaken to determine the permeability of Douglas bags to expired air, and it was found that appreciable amounts of carbon dioxide, oxygen, and nitrogen pass through their walls.

Investigators working with rubber have been familiar with its permeability to various gases. Hüfner[2] measured the solubility of gases in gray rubber and concluded that carbon dioxide was more readily absorbed than air, nitrogen, or hydrogen. Kayser[3] determined the solubility of carbon dioxide and hydrogen in rubber and found that the amount of gas dissolved increases as the pressure increases, and that the solubility is inversely proportional to the temperature. Also, in studying the passage of gases through a rubber membrane, he found that an increase in temperature caused an increase in permeability. Mitchell[4] long ago stated that rubber was permeable to gases, and Graham showed that carbon dioxide passed through a rubber membrane more rapidly than did air,[5] and that oxygen permeated more rapidly than nitrogen.[6]

Van Amerongen[7, 8] discussed in detail the theoretical and experimental aspects of the passage of gases through rubber membranes, and stated that a gas first dissolves in rubber to a degree which is proportional to its partial pressure, in accordance with Henry's law. It then diffuses in the rubber toward that area where the gas is present in lower concentration, in accordance with the law of Fick. Finally the gas passes out of the other surface of the rubber.

In his monograph on the methods of determining respiratory exchange, Carpenter[9] gives measurements of the rate of diffusion of carbon dioxide through the walls of a Douglas bag. During the short collection periods of five to ten minutes the carbon dioxide loss was negligible, but its concentration decreased 0.16 per cent after the expired air had remained in the bag

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for three and one-third hours. No other work dealing specifically with the measurement of the rates of passage of gases through the walls of the Douglas bag was found.

It was the purpose of this study to investigate the permeability of Douglas bags to carbon dioxide, oxygen, and nitrogen by measuring the changes in concentration of these gases under various conditions.

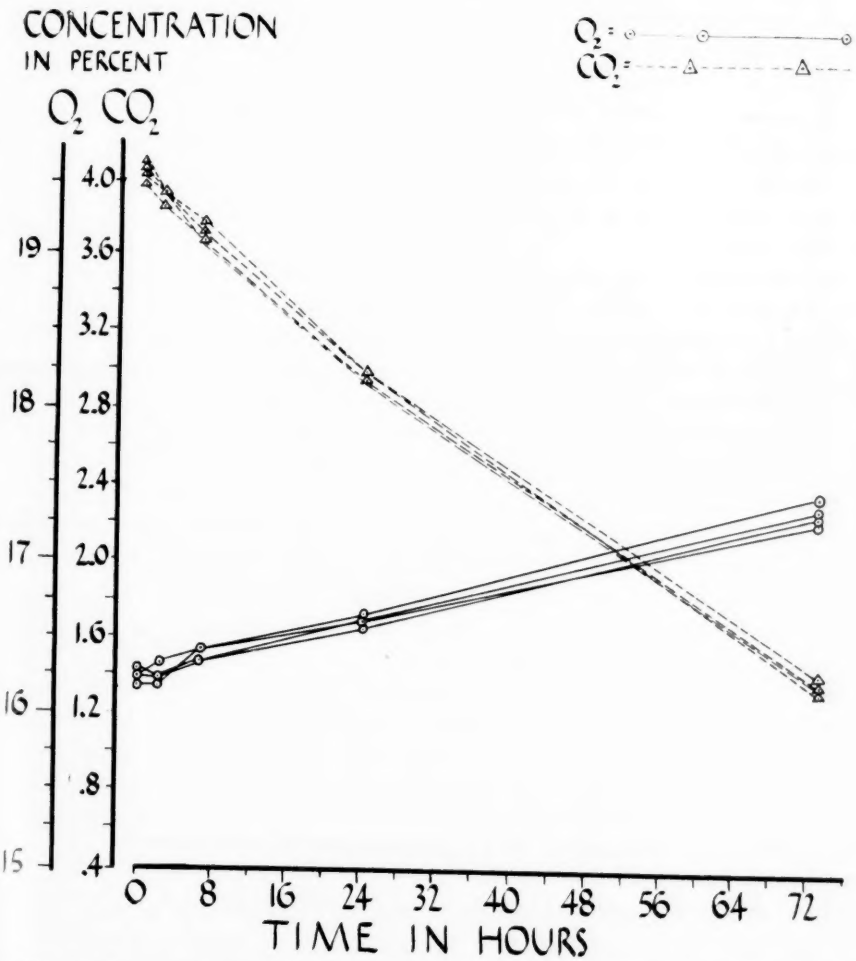

Fig. 1.—Changes in concentration of carbon dioxide and oxygen in each of four Douglas bags initially containing 22.3 liters of expired air. Note that the rates of decrease in carbon dioxide concentration and increase in oxygen concentration are almost identical in all four bags.

METHODS

Douglas bags* are made of natural rubber mixed with inorganic fillers, and have a canvas support on the outside. The gas is contained in them at nearly atmospheric pressure up to their capacity. They are available with capacities ranging from 30 to 200 liters. We used four bags of 100 liters capacity which had been in use in the laboratory for over two years, and a new bag of 100 liters capacity. All experiments and measurements were

done at room temperature (about 20° C.) and at ambient pressure.† At various intervals samples were taken from the Douglas bags and analyzed by the Scholander-Roughton method.[10] Gas volumes were measured with a "Precision" wet test meter. The method of emptying the bags by folding them was identical in all instances.

RESULTS

Comparison of the Permeability of Four Different Douglas Bags to Carbon Dioxide and Oxygen.—Four Douglas bags were filled with the same volume of expired air and the rate of change in concentration of carbon dioxide and oxygen determined over a period of seventy-two hours. The results of an experiment in which the initial volume (S.T.P.) was 22.3 liters, the carbon dioxide concentration 4.07 per cent, and the oxygen concentration 16.28 per cent, are shown in Fig. 1. It can be seen that the rate of decrease in carbon dioxide concentration and the rate of increase in oxygen concentration are almost identical in all four bags.

The experiment was then repeated twice: first, using a volume of 11.1 liters of expired air, with a carbon dioxide concentration of 3.51 per cent and an oxygen concentration of 17.14 per cent; and second, using a volume of 26.1 liters, the carbon dioxide concentration being 1.93 per cent and the oxygen concentration 19.52 per cent. Similar results were obtained in all instances: the rates of change in the concentrations of carbon dioxide and oxygen in the four bags were identical within the limits of analytical error.

This uniform permeability of several Douglas bags to carbon dioxide and oxygen indicates that their area, thickness, and composition are very similar. As illustrated in Fig. 5, the permeability of a new Douglas bag was almost identical with one which had been in use for more than two years.

Factors Affecting the Rate of Change in Concentration of the Components of a Gas Mixture.—Various initial volumes and concentrations of carbon dioxide and oxygen were introduced into five Douglas bags, and samples were removed and analyzed at different time intervals (Fig. 2). From a study of the graph of these data, it was evident that at least three factors were of importance in affecting the rate of change of the concentrations of carbon dioxide and oxygen: (a) the nature and properties of the gas, (b) the partial pressure gradient of each gas between the inside of the Douglas bag and the atmosphere, and (c) the initial total volume of the gas mixture.

(a) *The nature and properties of the gas:* When the partial pressure gradients were essentially the same, the slopes of the curves representing the rates of decrease in concentration of carbon dioxide were much steeper than the slopes of the curves showing the rate of increase in oxygen concentration, indicating that the bag is more permeable to carbon dioxide than to oxygen (Fig. 2). When the initial volume was 22.3 liters, the carbon dioxide decreased 1.05 per cent in concentration, while oxygen increased 0.36 per cent in concentration in twenty-four hours.

*The Douglas bags employed in these experiments were manufactured by the Devol Rubber Company.

†The mean atmospheric pressure in Denver is 628 mm. Hg.

The increase in concentration of oxygen is due both to the passage of oxygen into the bags from the atmosphere and to a decrease in the total volume of the gas mixture resulting from the loss of carbon dioxide. Similarly, the decrease in concentration of carbon dioxide is due in part to dilution of the total gas volume by oxygen entering the Douglas bag from the atmosphere.

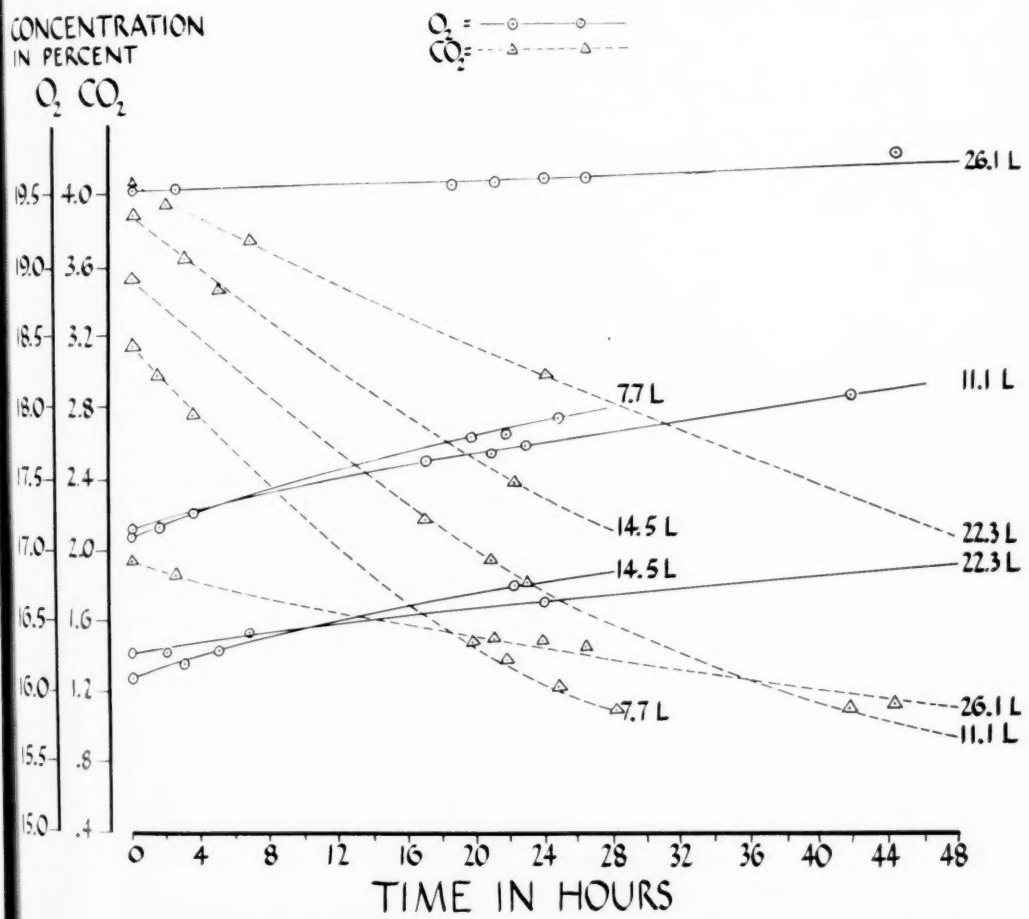

Fig. 2.—Changes in concentration of carbon dioxide and oxygen in five Douglas bags, each containing a different initial total volume of expired air. In each instance, carbon dioxide and oxygen are at different initial partial pressures. The rates of decrease in carbon dioxide concentration and increase in oxygen concentration are greater the smaller the initial total volume and the higher the initial concentration of the gases in the mixture.

In order to determine the relative importance of these two factors with regard to oxygen, the carbon dioxide in expired air was absorbed with soda lime and different volumes of the resulting mixture of oxygen and nitrogen were put into five Douglas bags and analyzed at intervals for oxygen. Since the permeability of rubber to oxygen is greater than to nitrogen (see below), the changes in oxygen concentration are predominantly due to the passage of this gas into the Douglas bag from the atmosphere. In all five instances the oxygen

concentration increased. The curve showing the rate of increase in oxygen concentration in 22 liters of such a mixture is shown in Fig. 3, and for comparison a curve for 22.3 liters of complete expired air containing carbon dioxide, oxygen, and nitrogen is also shown. It is evident from the slopes of these curves that the rate of increase in oxygen concentration is less when carbon dioxide is not present than when it is present. In twenty-four hours the oxygen in the

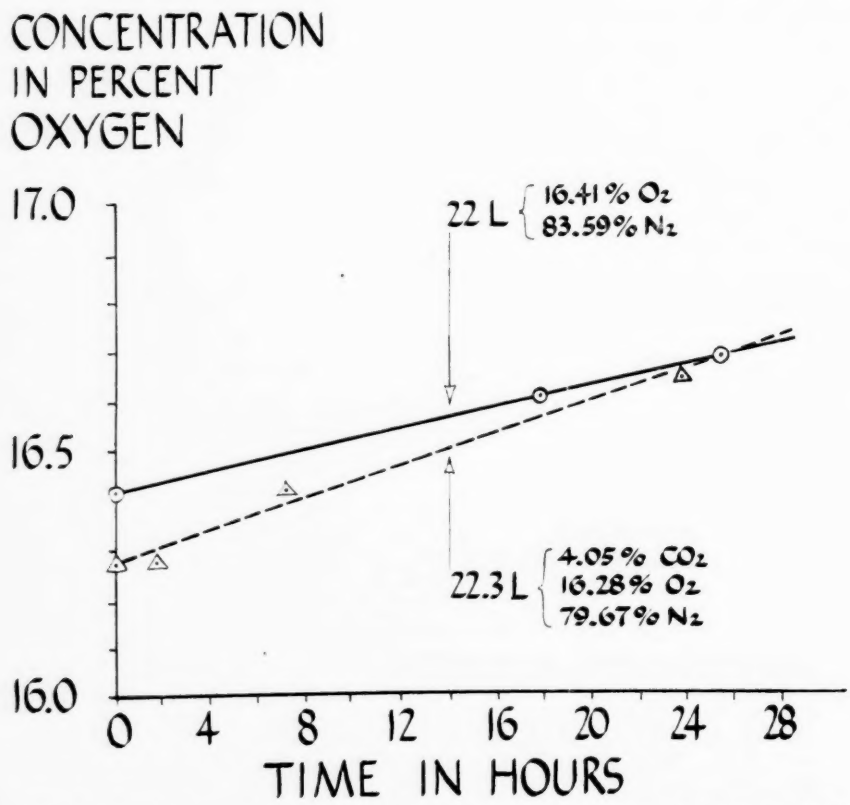

Fig. 3.—Comparison of the rate of increase in the concentration of oxygen in 22.3 liters of expired air containing carbon dioxide with the rate of increase in 22 liters of expired air from which the carbon dioxide has been absorbed. Due to the loss of carbon dioxide from the Douglas bag, the rate of increase in oxygen concentration is greater in the expired air containing carbon dioxide.

first mixture (oxygen and nitrogen) increased 0.25 per cent in concentration, while the oxygen in the second mixture (carbon dioxide, oxygen, and nitrogen) actually increased 0.36 per cent in concentration. It was calculated in the latter instance that of the 0.36 per cent increase in oxygen concentration, 0.19 per cent was due to oxygen entering the Douglas bag, and 0.17 per cent was due to the relative concentration of oxygen due to the loss of carbon dioxide from the bag.

The concentration of nitrogen in expired air is almost equal to that in the atmosphere, and the pressure gradient is quite small. Therefore, in the fore go-

ing calculations it has been assumed that the nitrogen concentration remained comparatively constant.

In order to determine the rate of passage of nitrogen through the walls of the Douglas bag, four of them were filled with different volumes of tank oxygen and allowed to stand, samples being taken at intervals and analyzed for nitrogen. The original concentration of nitrogen was less than 1 per cent, and there were therefore large gradients between the nitrogen levels in the bags and the nitrogen level in the atmosphere (Fig. 4). The composition of the atmosphere has

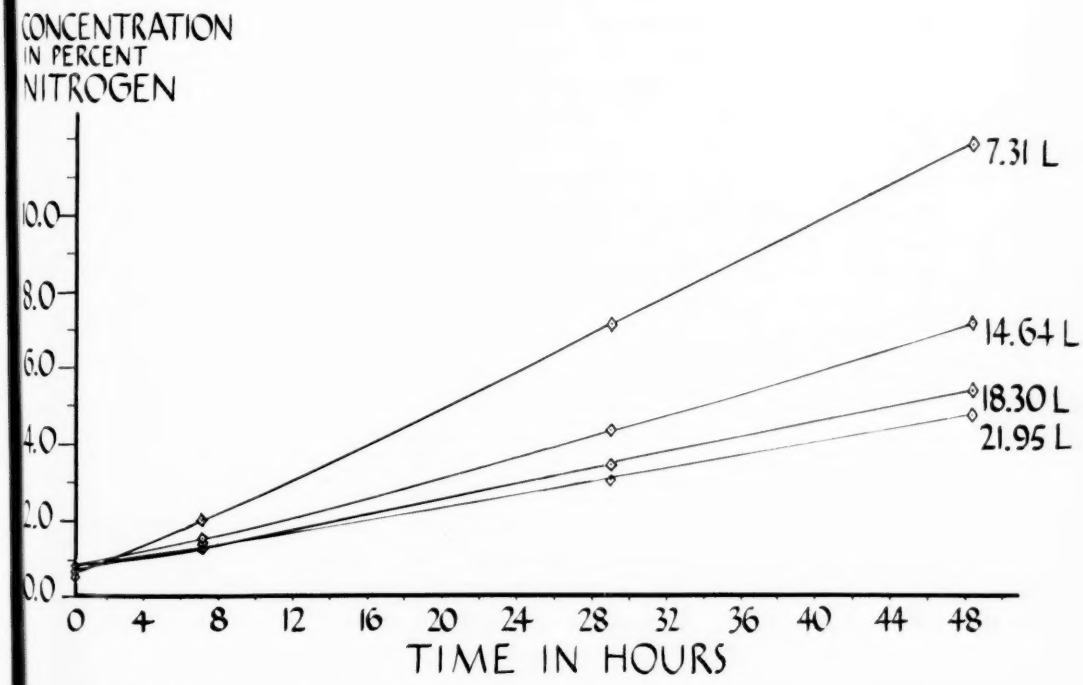

Fig. 4.—Increase in the nitrogen concentration in four Douglas bags, each of which contained a different initial volume of tank oxygen. There is a continual increase in the nitrogen concentration in the bags as nitrogen enters from the atmosphere, the rate of change being greater the smaller the initial volume of the gas. These data have been corrected for the decrease in volume due to the loss of oxygen from the bags into the atmosphere.

been taken as 0.04 per cent carbon dioxide, 20.93 per cent oxygen, and 79.03 per cent nitrogen. The passage of nitrogen into the bags over a period of time was appreciable, and the smaller the initial volume in the bags the greater was the rate of increase in the nitrogen concentration. When the volume of oxygen was 21.95 liters, the nitrogen increased from a concentration of 0.75 per cent to 1.29 per cent at the end of seven hours, and to 4.72 per cent at the end of forty-eight hours. Because of the high oxygen pressure gradient, a significant amount of oxygen escapes from the bags. This is an important factor contributing to the increase in concentration of nitrogen. The changes in nitrogen concentrations shown in Fig. 4 are due only to nitrogen entering the Douglas bags,

as corrections have already been made for the increase in nitrogen concentration due to the escape of oxygen. The average decrease in volume, mainly due to the loss of oxygen, was found to be 30 ml. per hour. This was determined by allowing various volumes of oxygen to remain in Douglas bags for seventy-six hours and measuring the changes in the volumes.

(b) *The partial pressure gradients of gases making up a mixture affect their rates of permeation:* The greater the difference in pressure of a gas between the atmosphere and the inside of the Douglas bag, the more rapid will be the change in its concentration. This can be seen (Fig. 2) by comparing the rates of change of carbon dioxide and oxygen in a mixture where the gradients are large (22.3 liters of expired air containing 4.05 per cent carbon dioxide and 16.28 per cent oxygen) with nearly the same volume of gas where the gradients are smaller (26.1 liters of expired air containing 1.93 per cent carbon dioxide and 19.52 per cent oxygen). In twenty-four hours, there was a decrease of 1.05 per cent in the carbon dioxide concentration and an increase of 0.36 per cent in the oxygen concentration in the first mixture, while in the second mixture the carbon dioxide decreased 0.44 per cent and the oxygen increased 0.10 per cent in concentration.

(c) *The rate of change in concentration of carbon dioxide and oxygen depends also upon the initial total gas volume:* Volumes of expired air ranging from 7.46 to 29.9 liters were put into four Douglas bags. For comparison, a new Douglas bag was filled with the same volume of expired air as one of the older bags (7.46 liters). A graphical presentation of these data (Fig. 5) shows that the rates of decrease in the carbon dioxide concentration and increase in the oxygen concentration are greater when the initial volume is small, and are smaller when the initial volume is larger. The four Douglas bags had essentially the same surface area, and the initial pressure gradient for each of the components of expired air was the same; therefore, the absolute amounts of carbon dioxide lost and oxygen gained were initially the same. The loss of the same volume of gas, however, produces a greater change in the concentration of the components of a gas mixture when the initial volume is small than when it is large.

DISCUSSION

The rate of passage of a gas through a membrane depends upon the characteristics of the gas and the membrane, the pressure gradient of the gas, and the temperature. A gas first dissolves in the membrane in proportion to its partial pressure,[7, 8] in accordance with Henry's law which states that at a constant temperature, the solubility of a gas varies directly with the pressure. It then diffuses in the membrane toward that area where the gas is present in lower concentration, in accordance with the law of Fick, which states that the rate of diffusion of a substance is equal to the product of the concentration gradient, the area, and the coefficient of diffusion of that substance in the particular solvent. Finally the gas escapes from the outer surface (see Equations, equation 1).

If unit values are taken for the pressure gradient, the area, and the thickness, then in unit time the quantity of a gas (Q) diffusing through this membrane is given by:

$$Q = Dh$$

where "Q" is the permeability coefficient, "D" is the diffusion coefficient, and "h" is the solubility coefficient. "Q" represents the cubic centimeters of gas at 0° C. and 760 mm. Hg which during one second penetrate one square centimeter of surface of the membrane, which is one centimeter thick, when the pressure gradient is 760 mm. Hg. "D," the diffusion coefficient, is numerically equivalent to the quantity of gas diffusing across a plane of unit area when the

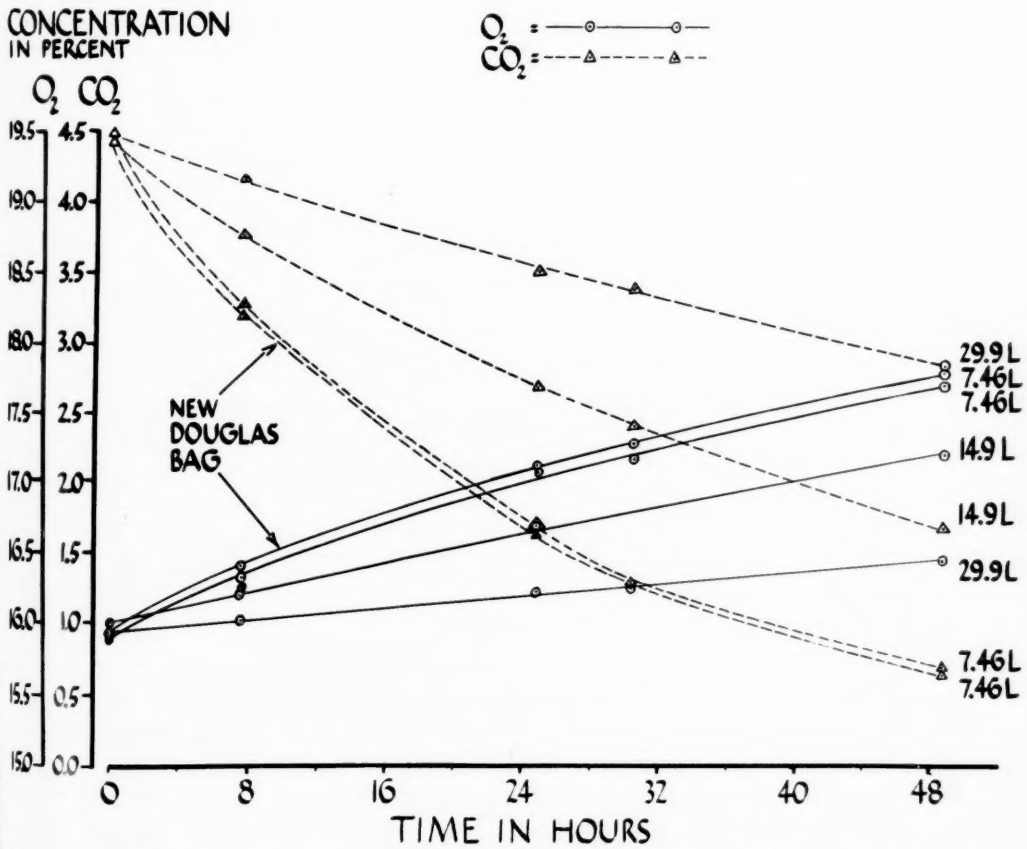

Fig. 5.—The four Douglas bags contained different initial volumes of expired air of the same composition. The rates of decrease in carbon dioxide concentration and increase in oxygen concentration are greater the smaller the initial total volume of the gas mixture. The permeability of a new Douglas bag was almost identical with that of one more than two years old.

concentration gradient is unity. The Bunsen solubility coefficient "h" represents the cubic centimeters of gas at 0° C. and 760 mm. Hg that dissolves in one cubic centimeter of the substance.

The permeability of a membrane to a gas is thus determined by both the solubility of the gas in the substance of the membrane and the rate of diffusion of the gas through that membrane.

The solubility of a gas depends upon the characteristics of the membrane, the nature of the gas, and the temperature. Natural rubber will dissolve a

greater quantity of carbon dioxide, oxygen, and nitrogen than various synthetic rubber substitutes. Materials added to rubber, such as sulphur, alter its permeability. The nature of the gas is an important factor, as is demonstrated by the fact that carbon dioxide is nine times more soluble in rubber than is oxygen; one milliliter of rubber at 25° C. dissolves 0.90 ml. of carbon dioxide, but only 0.099 ml. of oxygen.[8] That the temperature affects solubility is shown in the data of van Amerongen,[8] who found that oxygen, nitrogen, and hydrogen increase in solubility with an increase in temperature, while more soluble gases like carbon dioxide show a decrease in solubility as the temperature increases.

The rate of diffusion of a gas depends upon the nature of the gas and the character of the membrane. The larger the molecular weight of the gas, the slower its rate of diffusion. Hydrogen diffuses two to three times as fast as oxygen, nitrogen, and carbon dioxide because of the smaller diameter of its molecule. For natural rubber at 25° C., the diffusibility (D) of carbon dioxide is 11, which is less than that of oxygen (D = 18) and of nitrogen (D = 12.5), indicating that carbon dioxide diffuses through rubber only about 60 per cent as fast as oxygen. In spite of the slower rate of diffusion of carbon dioxide in rubber, rubber is more permeable to carbon dioxide because the latter is nine times more soluble in it than is oxygen.[8]

As noted above, the nature of the membrane affects the rate of diffusion of a gas. In particular, the addition of inorganic fillers increases the effective thickness of rubber, causing the gas molecules to follow a broken path; fillers also reduce the effective surface of the membrane.

From the permeability coefficient (Q) at 25° C. for natural rubber given by van Amerongen,[8] the ratio of the rates of permeation of nitrogen, oxygen, and carbon dioxide are 1:2.7:15.5. Our data for the Douglas bag indicate that the rates of permeation of nitrogen, oxygen, and carbon dioxide are in the ratio of 1:2.5:16.2, and are therefore in good agreement with van Amerongen's ratios.

On the basis of data which we obtained, formulas were developed (see Equations) which predict with accuracy the changes in concentration which have occurred after a gas mixture has remained in a Douglas bag for a known length of time. By knowing the total volume and pressure gradients of a mixture of gases after the latter has remained in the Douglas bag for a certain number of hours, the original concentrations of the component gases can be calculated. These formulas have been tested by experiments in which the initial concentrations were actually measured as well as later calculated from subsequent measurements by use of the formulas. Thus, the formulas may be used to correct changes in concentration of the component gases of a mixture which unavoidably has to be left in the bag for an appreciable length of time.

The significance and application of the above data in physiologic studies need not be stressed. Oxygen consumption and respiratory quotient determinations may be affected significantly if expired air remains in the Douglas bags for any length of time. Even slight changes in the concentration of carbon

dioxide and oxygen may significantly affect the R.Q. determination, because these changes are in an opposite direction. For example, in one instance the R.Q. of the expired air of a patient was calculated to be 0.76 when a sample was taken for analysis immediately after the collection; however, it was calculated to be 0.61 when the sample was taken after the expired air had remained in the Douglas bag for twenty-two hours.

The diffusion of nitrogen through the walls of the Douglas bag is appreciable when the gradient is large (Fig. 4). When 100 per cent oxygen is placed in a Douglas bag, it will diffuse out into the atmosphere, and nitrogen will pass into the bag, favored by the large gradients present. The calculation of the residual air volume by the Darling-Cournand open circuit method[11] is based on the mass of nitrogen washed out of the lungs by the breathing of oxygen for seven minutes. Unless samples for analysis are taken within a short interval after the collection of the expired gas, it is evident that the passage of nitrogen into the Douglas bag will increase the mass of nitrogen, resulting in an apparent increase in the residual air volume. In one instance, experiment showed that the $\frac{\text{residual capacity}}{\text{total capacity}}$ per cent ratio was increased by 5 per cent when the gas mixture remained standing in the Douglas bag for fifteen hours.

In general, the permeability of rubber to gases increases with an increase in temperature.[12] Edwards and Pickering[13] found this to be true for carbon dioxide, hydrogen, and helium, and van Amerongen has found that permeability is directly proportional to the temperature for hydrogen, helium, oxygen, nitrogen, and carbon dioxide. In our experiments the temperature could not be controlled closely, and all of them were carried out at room temperature (about 20° C.). No data concerning the effect of slight variations of temperature around the 20° C. level on permeability were obtained. Using the formula given by van Amerongen,[8] it has been calculated that there is an increase in permeability of 3.7 per cent for carbon dioxide, 4.0 per cent for oxygen, and 5.6 per cent for nitrogen when the temperature is raised from 20° C. to 21° C. Under certain conditions, therefore, the temperature effect on permeability may be significantly large.

The permeability of rubber to water vapor may be significant, for it is relatively high. Edwards and Pickering[10] have stated that rubber is about fifty times more permeable to water vapor than to hydrogen. Douglas bags must then also be permeable to water vapor, although no studies of this have been carried out. However, the $\frac{\text{residual capacity}}{\text{total capacity}}$ per cent ratio and the R.Q. determination would not be affected by a loss of volume due to the escape of water vapor from the Douglas bags.

Although natural rubber has proved quite permeable to nitrogen and oxygen, and particularly to carbon dioxide, certain synthetic rubber substitutes have lower permeabilities to these gases. Thiokol-B[8] is approximately one-fortieth as permeable to carbon dioxide and one-eightieth as permeable to oxy-

gen as is natural rubber. Neoprene-G is also much less permeable than rubber. Therefore, it is possible that Douglas bags made of these substitutes would permit longer periods of storage of gases with less chance of changes in concentration in their contents.

SUMMARY

The permeability of Douglas bags made of natural rubber to the carbon dioxide, oxygen, and nitrogen in expired air has been determined.

There is a continual loss of carbon dioxide from the bags, and a gain of oxygen from the atmosphere, resulting in an increase in the oxygen concentration. The permeability of the Douglas bag to carbon dioxide was found to be approximately six times greater than to oxygen. These changes in concentration of oxygen significantly affect the values for the R.Q. and B.M.R. when expired air remains in the bags for several hours before samples are taken for analysis.

Nitrogen also permeates through the walls of Douglas bags, although at a slower rate than carbon dioxide and oxygen. Significant errors in the determination of the residual air volume may occur when made by the open circuit method if the gas mixture is allowed to remain in the Douglas bag for a number of hours.

The rate of change in concentration of a gas in a Douglas bag depends upon the nature of the gas and the characteristics of the membrane, the pressure gradient, and the initial volume of the gas. The permeability of different Douglas bags of the same size is so uniform that formulas have been developed by which one can calculate the original concentrations of a gas from the total volume and composition of the gas mixture after it has remained in the Douglas bag for a known number of hours.

Synthetic substances of much lower permeability to carbon dioxide and oxygen could be used in making the Douglas bags.

EQUATIONS

The change in concentration of a gas in a mixture remaining in a Douglas bag at room temperature for a period of time depends upon the total initial volume of the gas mixture, the time the latter remains in the bag, the pressure gradient of the gas between its level in the bag and that in the atmosphere, the thickness and area of the wall of the bag, and the diffusibility and solubility coefficients of the gas for natural rubber at room temperature.

These relationships have been combined in a general form by van Amerongen[7, 8] as follows:

$$q = D \cdot h \cdot \frac{A}{d} (p_1 - p_2)\, t \qquad (1)$$

q = volume in ml. of gas that has permeated
D = coefficient of diffusion
h = coefficient of solubility
A = area of membrane in square centimeters
d = thickness of membrane, in centimeters
p_1 and p_2 = partial pressure of gas on the two sides of the membrane, in atmospheres
t = time of permeation, in seconds

Substituting concentrations for partial pressures:

(2) $q = D \cdot h \cdot \frac{A}{d} \cdot P\ (C - C_A)\ t$

(3) $q = K P\ (C - C_A)\ t$ where

C = concentration, in per cent, of gas in Douglas bag at time of sampling
C_A = concentration, in per cent, of gas in atmosphere
P = atmospheric pressure in atmospheres
$K = D \cdot h \cdot \frac{A}{d}$, where the constants are expressed as follows:
"q" in liters, and
"t" in hours

The change in per cent of gas permeating is $\Delta C = \frac{q}{V}$ over a period of time (Δt), where

V = total initial volume of gas which is assumed to remain constant, the small change not being significant.

(4) $\frac{q}{V} = \frac{K P}{V}\ (C - C_A)\ \Delta t = \Delta C$

$$\frac{\Delta C}{\Delta t} = \frac{K P}{V}\ (C - C_A)$$

After integrating and changing logarithms from base "e" to base "10":

(5) $\log\left(\frac{C - C_A}{C_0 - C_A}\right) = \frac{K'Pt}{V}$

where C_0 = concentration in per cent of the gas in the Douglas bag at zero time

For Carbon Dioxide: Since the amount of CO_2 in the atmosphere is very small (0.04 per cent), it may be neglected.

(6) $\log\left(\frac{C_{co_2}}{C_{o\ co_2}}\right) = \frac{K_{co_2} Pt}{V}$

C_{co_2} = concentration in per cent of CO_2 in bag at time of sampling
$C_{o\ co_2}$ = initial concentration in per cent of CO_2 in bag
K_{co_2} = -.156 from our data on five different Douglas bags

For Oxygen: A. The change in concentration of oxygen in the Douglas bag due to oxygen permeating into it from the atmosphere is:

(7) $\log\left(\frac{C_{o_2} - 20.93}{C_{o\ o_2} - 20.93}\right) = \frac{K_{o_2} Pt}{V}$

C_{o_2} = concentration in per cent of $_{o_2}$ in bag at time of sampling
$C_{o\ o_2}$ = initial concentration in per cent of $_{o_2}$ at the time of collection of the gas in the Douglas bag
K_{o_2} = -.024 from our data on five different Douglas bags

B. The change in concentration of oxygen in the Douglas bag due to the escape of carbon dioxide into the atmosphere:

$$V\ (C_{o\ o_2}) = (V + \Delta V)\ (C_{o_2} + \Delta C_{o_2})$$

but $\Delta V = \left(\frac{\Delta C_{co_2}}{100}\right) V.$

(8) $\Delta C_{o_2} = -0.01\ (C_{o_2})\ (\Delta C_{co_2})$

The changes in concentration of oxygen calculated by means of equations (7) and (8) are then added to find the total change in the oxygen concentration.

CALCULATION OF PERMEABILITY (Q) CONSTANTS

Since $Q = D \cdot h$, the "Q" values have been calculated by substituting in equation (2) the various known quantities for the Douglas bag, and then solving for "Q." The permeability constants for natural rubber at 21° C., with van Amerongen's[7, 8] values for comparison, are:

			VAN AMERONGEN	
	"Q"	RATIO Q/Q_{N_2}	"Q"	RATIO Q/Q_{N_2}
CO_2	60.0×10^{-8}	16.2	87.0×10^{-8}	16.4
O_2	9.2×10^{-8}	2.5	15.0×10^{-8}	2.8
N_2	3.7×10^{-8}	1.0	5.3×10^{-8}	1.0

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THE METABOLISM OF WATER AND ELECTROLYTES IN CONGESTIVE HEART FAILURE

I. The Electrolyte and Water Content of Normal Human Skeletal Muscle

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IN RECENT years the concept of the pathogenesis of congestive heart failure has undergone an extensive revision.[1, 2] Attention has been focused on extracardiac factors such as altered renal function, antidiuretic hormone secretion, adrenal cortical influences, and the osmotic relationships of intra- and extracellular water.[3] Data derived from balance experiments during recovery from congestive failure have suggested that intracellular electrolyte content is decreased during the development of heart failure.[4-6] Recent studies of the cation content of bone have demonstrated that a significant portion of the skeletal electrolyte is exchangeable, thus challenging the results obtained from the usual type of balance experiments.[7] Several groups of workers have undertaken the direct analysis of skeletal and cardiac muscle in heart failure in order to document the changes in tissue electrolytes.[8-12] The earlier workers failed to consider the fat content of skeletal muscle.[9] In a more recent report the fat content for skeletal muscle has been assumed to be one per cent, and, as a result, these observations are not strictly comparable with other published values.[10] Still another group used alcohol-ether for the extraction of fat in the tissues analyzed and, therefore, their values for the electrolyte and water content of skeletal muscle would tend to be high.[11]

Several studies have included values for normal human skeletal muscle.[10, 11, 13] One of these recorded results on tissues obtained at autopsy and included no simultaneous measurements of serum electrolytes.[10] The method for handling the fat content of the tissues analyzed varied in each of these reports. As a result the available values for the electrolyte and water content of normal human skeletal muscle are of limited value for comparative purposes.

This study was undertaken to evaluate the changes in tissue electrolyte concentrations in the edema of heart failure. Because of the paucity of published data on the electrolyte and water content of normal human skeletal muscle, the present series of observations in patients free of electrolyte abnormalities is being presented. A second report dealing with the findings in a series of individuals with heart disease before and after the delivery of peripheral edema will be presented. Also, the distribution of tissue electrolytes as correlated with metabolic balance data will constitute a later report.

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METHODS

Specimens of skeletal muscle were obtained from control subjects either at the time of abdominal surgery or under local procaine anesthesia. Tissues obtained during surgical operations were obtained early in the procedure so as to avoid the possible effects of prolonged anesthesia and large infusions of fluids. In the cases in which tissue was obtained under local anesthesia, the infiltrate of procaine was kept at a distance of at least ten centimeters from the actual biopsy site. A comparison of results obtained under general and regional block anesthesia showed no significant differences attributable to the anesthetic agent used.

The muscle specimens, weighing 4 to 10 Gm., were immediately placed in a tightly sealed jar and transported to the laboratory. Analyses were begun within five minutes after the specimen was obtained. All visible fat and connective tissues were dissected away and discarded. The samples were then lightly blotted with filter paper to remove excess blood and placed in a tared stoppered weighing bottle and thoroughly minced with scissors. Weighed aliquots were then transferred into Kjeldahl flasks, large test tubes, and tared silica beakers for chemical analyses. Duplicate samples of approximately 300 milligrams were used for nitrogen determinations, 1.0 Gm. for chloride determinations, and 2.0 to 3.0 Gm. for determination of water, neutral fat, sodium, and potassium. All values were corrected for neutral fat content. The fat-free weight was based on the average value for fat obtained from the specimens used for sodium and potassium analyses.

Total nitrogen was determined by the micro-Kjeldahl method, chloride by the Wilson and Ball modification[14] of the Van Slyke method.[15] The water content was determined by drying to constant weight, at 100° C., the samples of tissue in the silica beakers. Neutral fat was estimated by triple extraction of these dried tissues with ethyl ether.[16] After the determination of the fat content, the ether used in each extraction was returned to the respective sample and evaporated to dryness. Four milliliters of 4N sulfuric acid were added to each beaker and then placed in a 110° C. oven until the residue formed a thick syrupy material after which they were ignited overnight in a 500 to 525° C. muffle furnace. The cooled white residue was dissolved in one milliliter of concentrated nitric acid and then transferred quantitatively with water to a 10 ml. volumetric flask. After making to volume, aliquots of this solution were diluted to a suitable volume, incorporating Sterox SE* as a wetting agent for the sodium and potassium determinations. These determinations were carried out on a Weichselbaum-Varney flame spectrophotometer. The values for sodium and potassium obtained with this instrument were periodically checked against values from independent determinations by gravimetric and titrimetric methods as well as against values obtained with a Perkin Elmer internal standard flame photometer. The average error in any determination with the Weichselbaum-Varney instrument was less than 1 per cent for both sodium and potassium.

Venous blood drawn with minimal stasis in oiled syringes was obtained at the time the biopsy specimen was being removed. After approximately fifteen minutes the blood samples were centrifuged and the serum separated anaerobically under light mineral oil.

Serum water content was determined in duplicate by drying one milliliter to constant weight in a thermostatically controlled oven at 100° C. Serum chloride was determined by the Wilson and Ball modification[14] of the Van Slyke method,[15] sodium and potassium with the Weichselbaum-Varney flame spectrophotometer, carbon dioxide by the method of Van Slyke and Neill,[17] and pH with a Beckman pH meter.

CALCULATIONS AND SYMBOLS

Chloride was utilized as a reference for the estimation of the volume of extracellular water in the muscle specimens. The analytical data were subjected to the same method of calculation as used by Hastings and Eich-

*Sterox SE was generously supplied by The Monsanto Chemical Corp., St. Louis, Mo.

berger[18] to estimate the phase volumes of the skeletal muscle and the concentrations of potassium, sodium, and chloride in the extracellular fluid. The following symbols and formulas are used throughout:

$(H_2O)s$ = Serum water Gm./kg. serum
$(H_2O)m$ = Muscle water Gm./kg. fresh fat-free muscle
$(D)s$ = Density of serum Gm./ml.
$\{Cl\}f$ = Chloride, meq./kg. extracellular phase

$$\{Cl\}f = \frac{\text{Serum Cl meq./L.}}{(D)s \times (H_2O)s \times 0.95} \times 0.99$$

$(Cl)m$ = Chloride, meq./kg. fresh fat-free muscle
(F) = Gm. extracellular phase/kg. fresh fat-free muscle
(C) = Gm. intracellular phase/kg. fresh fat-free muscle
$(H_2O)f$ = Gm. extracellular water/kg. fresh fat-free muscle
$(H_2O)c$ = Gm. intracellular water/kg. fresh fat-free muscle
$\{H_2O\}c$ = Gm. intracellular water/kg. intracellular phase
$(K)c$ = Intracellular potassium meq./kg. intracellular water

$$(F) = \frac{(Cl)m}{\{Cl\}f} \times 1{,}000$$

$$(H_2O)f = (F) \times 0.99$$

$$(H_2O)c = (H_2O)m - (H_2O)f$$

$$\{H_2O\}c = \frac{(H_2O)c}{(C)}$$

$$(K)c = \frac{(K)m - (\{K\}f \times [H_2O]f)}{(H_2O)c}$$

RESULTS AND DISCUSSION

Table I lists the original data of the chemical analyses for the serum and skeletal muscle in the control subjects. Table II gives the estimated values for the extra- and intracellular water content and distribution of electrolytes. It will be noted that the estimated distribution of the sodium and potassium corresponds closely to previously published values for animal tissues.[18] When allowances are made for the method of estimating the fat content of fresh tissue, these values correspond to the normal values previously reported.[10, 11, 13] The wide variation in the fat content of the tissues reported again demonstrates the importance of fat determinations when tissue analyses are performed.

The validity of the estimated values obtained from the tissue analyses of this type is dependent upon the accuracy with which the volume of extracellulaar fluid can be estimated. A recent comparison of the inulin and chloride space in human skeletal muscle has revealed that the apparent volume of distribution of inulin is significantly smaller than the chloride space in normal individuals, whereas in edematous cardiacs the reverse is true.[11] In the normal this would support the concept that a portion of the tissue chloride is nondiffusible. In the present study chloride has been used throughout as the basis of reference for estimating the quantity of extracellular fluid in the tissues being analyzed. No correction has been made for nondiffusible chloride; however, a systematic correction for the water content of the extracellular phase has been incorporated into the calculations of extracellular water

utilizing a factor of 0.99 as representing the water content of this phase. If the actual volume of the extracellular phase is less than the chloride space, as

TABLE I. CONTROLS. ANALYSES OF SERUM AND TISSUES FROM PATIENTS

1 PT.	2 DX.	3 SITE	4 ANES.	5*	6 pH	7 CO_2 (mM./L.)	8 H_2O† (GM.)	9 Cl† (MEQ.)	10 Na† (MEQ.)	11 K† (MEQ.)	12 N† (GM.)	13 FAT (%)
McC.	*a*	*1*	*A*	S	7.38	28.2	907.9	93.8	136.6	4.70		
49				M			781.8	17.2	40.0	88.80	30.1	1.2
Skog.	*b*	*2*	*B*	S	7.42	25.2	916.4	100.5	136.8	4.02		
37				M			773.6	17.4	36.8	87.90	28.6	2.0
Dig.	*c*	*1*	*B*	S	7.41	24.0	911.6	101.2	138.4	4.02		
59				M			783.6	25.4	49.3	85.10	26.8	10.5
McN.	*b*	*2*	*C*	S	7.41	26.4	911.6	102.9	137.5	4.43		
53				M			777.0	14.6	31.1	99.80	29.6	5.6
Beef.	*b*	*2*	*B*	S	7.39	25.3	910.8	100.6	140.4	4.65		
52				M			779.6	23.0	36.9	94.30	25.2	4.0
Day.	*d*	*2*	*C*	S	7.45	26.4	903.7	100.5	140.9	4.90		
48				M			766.5	16.7	28.8	102.20	32.6	3.7
Malt.	*c*	*2*	*A*	S	7.43	24.5	909.8	101.0	134.7	3.78		
44				M			762.3	18.2	29.3	93.80	31.6	0.9
Gold.	*e*	*2*	*D*	S	7.40	24.3	910.6	101.4	140.5	4.49		
58				M			777.5	18.0	35.2	89.40	24.9	11.8
Red.	*c*	*2*	*E*	S	7.46	25.8	912.8	96.0	132.3	4.16		
49				M			775.7	16.8	35.4	92.9	29.8	5.3
Rose.	*d*	*2*	*C*	S	7.43	30.9	907.5	96.3	140.0	4.47		
43				M			783.7	18.7	29.4	91.8	31.3	1.8
Jac.	*d*	*2*	*B*	S	7.39	30.4	912.4	98.7	132.6	4.52		
62				M			774.5	19.5	28.1	96.6	27.9	2.5
Nels.	*b*	*2*	*B*	S	7.42	28.2	912.1	103.2	134.4	4.50		
51				M			779.2	27.1	42.0	88.90	31.5	6.0
Pears.	*e*	*2*	*A*	S	7.49	26.4	915.1	100.0	130.4	4.43		
38				M			782.4	14.4	22.0	107.1	30.6	10.4
Thot.	*b*	*2*	*B*	S	7.43	28.6	898.4	96.1	135.1	4.37		
58				M			784.7	25.3	36.2	89.00	30.3	6.6
Yam.	*f*	*3*	*F*	S	7.35	19.0	915.0	105.9	138.6	3.73		
29				M			770.2	14.9	29.6	94.70	30.1	0.4
Dom.	*c*	*2*	*A*	S	7.35	29.0	916.9	102.1	134.7	4.33		
37				M			733.6	18.5	28.7	102.00	31.5	1.1
Mean				S	7.41	26.4	910.8	100.0	136.5	4.34		
S. D.					0.04	2.8	4.6	3.0	2.5	0.31		
Mean				M			776.6	19.1	33.7	94.00	29.53	
S. D.							6.4	3.9	6.4	5.94	2.2	

Diagnoses (Column 2): *a*, empyema; *b*, cholelithiasis; *c*, carcinoma of colon; *d*, peptic ulcer; *e*, carcinoma of breast; *f*, convalescent rheumatic fever.

Biopsy site (Column 3): 1, latissimus dorsi; 2, rectus abdominus; 3, quadratus femorus.

Anesthesia (Column 4): *A*, pentothal; *B*, spinal; *C*, cyclopropane; *D*, NO_2-ether-oxygen; *E*, C_2H_4-O_2-ether-oxygen; *F*, local procaine.

*S = serum; M = muscle.

†All muscle values are expressed per kg. of fresh, fat-free muscle.

All serum values are expressed per kg. of serum.

suggested by the inulin values, then the volume of intracellular water would be correspondingly greater, and as a result the concentration of intracellular solutes would be lower than those recorded above. Our calculations led to the following results for the phases in the muscle: extracellular phase (F) 167 Gm. ± 33; intracellular phase 833 Gm. ± 33. These data were also used to calculate the amount of water in the intracellular phase $\{H_2O\}c$. (Table II.)

It is notable that while the over-all content of water and electrolytes in skeletal muscle exhibits a relatively small variation, the volume of extra- and intracellular water varies over a somewhat greater range. This is undoubtedly due to the variation in the individual state of hydration. Despite this

TABLE II. DERIVED VALUES BASED ON ANALYTICAL DATA

PATIENT	GM./KG. FRESH FAT-FREE TISSUE		GM./KG. CELLS	MEQ./KG. INTRACELLULAR WATER			RATIO:	
	$(H_2O)f$	$(H_2O)c$	$\{H_2O\}c$	(Na)	(K)	(Na + K)	$\frac{(Na)_m^*}{(Cl)_m^*}$	$\frac{(K)_m^*}{(N)_m\dagger}$
McC.	158.3	623.5	741.1	27.9	141.1	169.0	2.32	2.95
Skog.	150.7	622.9	733.8	25.0	140.2	165.2	2.12	2.81
Dig.	217.3	566.4	724.0	32.3	148.7	181.0	1.95	3.17
McN.	122.9	654.1	746.3	20.9	151.7	172.6	2.14	3.37
Beef.	197.8	581.8	725.6	14.1	160.4	174.1	1.62	3.75
Day.	142.7	623.8	728.0	12.7	162.7	175.4	1.71	3.12
Malt.	155.7	606.6	718.8	12.5	153.6	166.1	1.60	2.97
Gold.	153.6	623.9	737.8	20.7	142.2	162.9	1.95	3.59
Red.	151.3	624.4	736.1	23.7	147.7	171.4	2.11	3.11
Rose.	169.1	614.6	740.9	8.0	148.1	156.1	1.56	2.92
Jack.	171.7	602.8	728.4	7.6	158.9	166.5	1.44	3.28
Nels.	227.3	551.9	716.4	19.0	158.9	178.1	1.54	2.84
Pears.	125.1	657.3	752.4	7.9	162.0	169.9	1.53	3.49
Thot.	224.6	559.4	723.6	7.9	157.3	165.2	1.43	2.94
Yam.	122.3	646.9	738.5	18.9	145.6	164.5	1.98	3.65
Dom.	157.7	615.9	732.6	11.2	164.5	175.7	1.55	3.23
Mean	165.5	611.0	732.8	16.9	152.7	169.6	1.78	3.20
S. D.	33.3	30.8	9.6	7.6	7.9	6.2	0.3	0.3

*meq./100 Gm. dry fat-free solids.
†Gm./100 Gm. dry fat-free solids.

variance in the volume distribution of water in skeletal muscle, the constancy of the percentage of water of the intracellular phase in normal tissue is once again emphasized.[18] The combined concentration of the intracellular cations sodium and potassium is also relatively constant. These factors illustrate the ability of the normal organism to maintain a relatively constant intracellular environment despite variations in the distribution of water under normal conditions.

SUMMARY

The distribution of water and electrolytes in normal human skeletal muscle has been described in sixteen control subjects. The fat content of the tissues observed varied from 0.4 per cent to 11.8 per cent. When fresh muscle was corrected for its fat content in the tissues analyzed, one kilogram was found to contain 776.6 ± 6.4 Gm. of water; 19.1 ± 3.9 meq. of chloride; 33.7 ± 6.4 meq. of sodium; 94.0 ± 5.9 meq. of potassium; and 29.5 ± 2.2 Gm. of nitrogen. The extracellular phase of muscle contained 165.5 ± 33.3 Gm. of water and the intracellular phase 611.0 ± 30.8 Gm. of water. The percentage of water in the intracellular phase was 73.3 ± 1.0 per cent. The concentration of intracellular potassium was 152.7 ± 7.9 meq. per kilogram of intracellular water, that of sodium 16.9 ± 7.6 meq., and the combined sodium and potassium 169.6 ± 6.2 meq. per kilogram of intracellular water. The mean value for the potassium to nitrogen ratio was 3.20 ± 0.3, and the mean value for the sodium to chloride ratio was 1.78 ± 0.3.

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CEREBROSPINAL FLUID SODIUM VALUES IN ESSENTIAL HYPERTENSION

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THERE has been increasing evidence that an abnormal sodium ion metabolism is related to certain hypertensive states. Braun-Menendez[1] suggests that salt and water metabolism may have more clinical significance in regulation of normal blood pressure in the body than has been appreciated previously. Tobian and associates[2] showed that the human renal arteries from autopsy material of hypertensive patients contained a higher sodium content of the medial and internal layers than in normotensive patients. Likewise, these workers showed that the psoas muscle in hypertensive patients showed a similarly higher content of sodium than in normotensives.

Grollman[3] found an elevated sodium content in various viscera of animals made hypertensive for experimental purposes. Selye and co-workers[4] have demonstrated that sodium ion is necessary for the production of experimental hypertension with desoxycorticosterone administration.[4]

Recently, we have been investigating the sodium content of body fluids in hypertensive patients. Previously we reported a series of serum sodium determinations made on uncomplicated hypertensive patients as compared to that of the normotensives, and found them to be of significantly higher range on an average in hypertensives as compared to that of normal individuals.[5] Apparently investigation of the sodium content of other body fluids, such as the cerebrospinal fluid, has not been undertaken previously.

We have recently determined the sodium content of the cerebrospinal fluid in 28 hypertensive patients. The purpose of this paper is to present these findings, together with a correlation with those of a normotensive series.

MATERIALS AND METHODS

Subjects Study.—The outpatient department of the Jefferson-Hillman Hospital offered the source of the patients studied. In this clinic there are three times as many Negro admissions as whites; 26 Negro and 2 white hypertensive patients with diastolic blood pressure readings of 100 mm. or above were subjected to spinal fluid examinations. The ages ranged from 28 years to over 62 years of age, with a majority between 35 and 50 years.

The diagnosis of essential hypertension in these patients was arrived at merely on the basis of exclusion of known causes of hypertension, such as pyelonephritis or other primary renal diseases. A majority of these patients had been observed over a period of several years before the diagnosis of essential hypertension was substantiated. No cases of so-called "hormonal hypertension," as described by Schroeder, were examined. Spinal fluid

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examinations were made on patients who had had no therapy or in which it had been discontinued for at least thirty days before the examination was made.

Sodium concentration in cerebrospinal fluid of normal individuals has been reported previously.[6] These reported values were the basis for comparison in this hypertensive series.

Technique.—Cerebrospinal fluid samples were obtained from nonfasting patients routinely on admission to the clinic. A modified flame photometric technique, as described in our previous studies, was used.[6] Samples were analyzed in duplicate, and the average value of these readings were taken as the correct value.

The normotensive frequency distribution curve (Fig. 1) is based on previously reported normal findings.[6] In order to have a basis for comparison with the 28 hypertensive subjects, the number of subjects in each distribution group of the normotensive was multiplied by 28/100, giving the values plotted in Fig. 1.

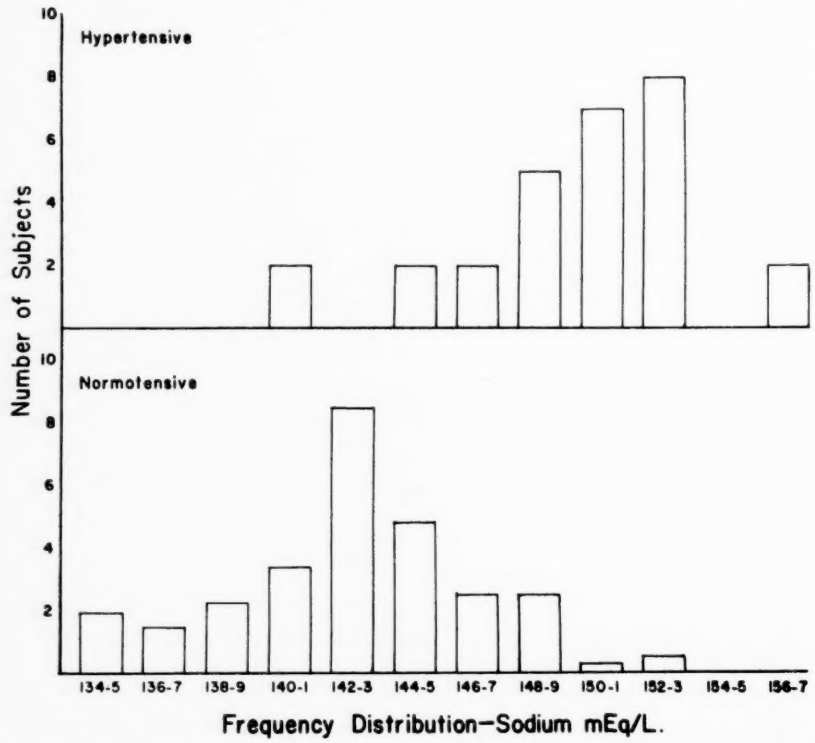

Fig. 1.—Frequency distribution curve of cerebrospinal fluid sodium values in hypertensives as compared with those of normotensive individuals.

RESULTS

The mean value for "normal" cerebrospinal fluid sodium content, as determined in our laboratory, was 142.27 meq. per liter. The mean value found in the small group of hypertensive patients was 149.6 meq. per liter, an increase of 7.13 meq. The standard deviation of the difference between the two means equals plus or minus 0.84. The number of standard errors contained within the difference between the two means equals 9.49. Therefore, the difference between the two

TABLE I

NORMALS		HYPERTENSIVES	
Mean, 100 specimens	142.47 meq./l.	Mean, 28 specimens	149.60 meq./l.
Mean, 10 highest values	149.30 meq./l.	Mean, 10 highest values	153.00 meq./l.
Mean, 3 highest values	151.30 meq./l.	Mean, 3 highest values	155.30 meq./l.
Highest value	152.00 meq./l.	Highest value	157.00 meq./l.
Lowest value	134.00 meq./l.	Lowest value	140.00 meq./l.
Mean, 10 lowest values	134.80 meq./l.	Mean, 10 lowest values	145.60 meq./l.
Mean, 3 lowest values	134.00 meq./l.	Mean, 3 lowest values	141.20 meq./l.

means is highly significant, and that the probability that the results were obtained by chance are practically nil.[7]

The standard deviation in the 100 normal determinations was plus or minus 3.99 meq. per liter, and the standard error was ± 0.399 meq. per liter. In the 28 hypertensive patients the standard deviation was plus or minus 3.88 meq. per liter, with a standard error of ± 0.73 meq. per liter.

In the frequency distribution curves shown in Fig. 1, it is immediately obvious that there is a marked tendency toward an increase in sodium values present in the cerebrospinal fluid of hypertensive individuals. Table I shows comparative mean values.

The cerebrospinal potassium values determined at the same time compared favorably with those of the normal series.

DISCUSSION

These findings offer further evidence of abnormal sodium retention in the hypertensive state. One might be tempted to conclude that these findings might offer further support to the concept of abnormal activity of the adrenal gland in the production of the hypertensive syndrome. It might be wise, however, to avoid such a conclusion for the time being, but merely to present them as further evidence of abnormal sodium metabolism in this disease state.

CONCLUSIONS

A series of cerebrospinal fluid sodium determinations made on uncomplicated hypertensive patients has been presented. These values were found to be higher than those in a series of 100 normotensive subjects. We have intentionally avoided any conclusions at this time as to the significance of these findings.

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LABORATORY METHODS

PURIFICATION AND STABILIZATION OF INFLUENZA VIRUS ANTIGENS FOR USE IN HEMAGGLUTINATION-INHIBITION OR COMPLEMENT FIXATION ANTIBODY TITRATIONS

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THE chick cell agglutinating activity (CCA) of crude allantoic fluid infected with influenza virus deteriorates rapidly under storage at 4° C. This loss of activity is apparently associated with the precipitation of the nonviral proteins which comprise more than 90 per cent of the total protein present.[1] In an effort to remove nonviral protein, Cox and his associates precipitated the fluid with methanol and resuspended the virus in a 0.1 molar sodium and potassium phosphate buffer. A product with satisfactory CCA was obtained. In later work, however, the results with the 0.1 molar buffer were not reproduced, and better yields of virus were obtained when a 0.3 molar sodium and potassium phosphate buffer was used as the eluting and suspending agent. The LEE strain of influenza virus was precipitated optimally by a final methanol concentration between 20 and 30 per cent. A 21 to 23 per cent concentration of methanol gave the best yields of the PR-8 strain.[2, 3]

In this laboratory, extension of this work was undertaken in an effort to obtain a purified and stable virus suspension for routine use in serologic tests for the diagnosis of influenza.

MATERIALS AND METHODS

The influenza virus strains PR-8, FM-1, and LEE employed in our laboratories in the serologic diagnosis of influenza were obtained from Herald R. Cox,* and since then have been propagated at this unit in embryonated eggs.

At the time of use in this study, the PR-8 strain had been passed through 198 ferret, 698 mouse, and 30 egg passages (PR-8, $F_{198}M_{698}E_{30}$). The FM-1 and LEE strains had the following passage formulas, respectively: FM-1 M_8E_{22}, and LEE, $F_8M_{137}E_{83}$.

The precipitating agents employed were acetone-free absolute methanol (ACS), having an assay of 99.5 per cent purity; and 95 per cent ethanol (ACS).

In an effort to diminish denaturation, the temperature of the infected allantoic fluid was kept at 0° C. to 2° C., and the methanol at −55° C. to −60° C. Inside and outside temperatures of the receiving and dispensing containers were checked at the beginning

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*Lederle Laboratories, Pearl River, N. Y. (1949).

and throughout the addition of methanol. Methanol was added to the infected allantoic fluid at a rate of 10 ml. per minute with continuous mechanical agitation. After the addition of methanol the mixture was allowed to stand for three hours at −4° C.

The methanol-allantoic fluid mixture was then centrifuged at 3,500 r.p.m. for thirty minutes at −5° C. The supernatant was decanted and discarded. The virus was eluted from the sediment with the buffer solutions described later for thirty minutes at 20° to 24° C.

After thirty minutes of elution, the buffered eluate was spun at 1,500 r.p.m. for fifteen minutes at 20° to 24° C. The supernatant containing the virus was saved and stored in a sterile glass screw-capped bottle at 4° C. Merthiolate to a final concentration of 1:10,000 was added as a preservative. The sediment was discarded.

Viral activity of the resulting suspension was measured by the method of Hirst and Pickels.[4]

RESULTS

Selection of the Optimal Precipitating Agent.—Using strain PR-8, a comparison was made between methanol, ethanol, and mixtures of the two at 25 per cent increments by volume as precipitating agents.

It was observed that when the percentage of ethanol in the precipitating agent was increased, there was a progressive increase in the rapidity of precipitation. Twenty-five per cent ethanol in the precipitating agent caused rapid precipitation and gave an adequate yield of processed virus (Table I). When the organic precipitating agent consisted of more than 25 per cent ethanol, the recovery of virus, or at least the CCA activity of the virus suspension, was greatly inhibited. During three weeks of storage at 4° C., marked differences were observed in the stability of the CCA titer of virus suspension that were processed similarly with the several precipitating mixtures. As seen in Table I, the chicken cell agglutination titer in suspensions prepared by precipitation with methanol alone were more stable upon storage than were those prepared by methanol—ethanol precipitation.

TABLE I. CHICKEN CELL HEMAGGLUTINATION TITERS OF ANTIGEN ELUTED AND SUSPENDED IN 0.1M SODIUM AND POTASSIUM PHOSPHATE BUFFER, pH 7.0 FROM PRECIPITATES OBTAINED BY TREATMENT OF ALLANTOIC FLUID INFECTED WITH PR-8 INFLUENZA VIRUS (ORIGINAL TITER 142) WITH VARIOUS MIXTURES OF METHANOL AND ETHANOL

PRECIPITATING AGENT* (PER CENT)		HEMAGGLUTINATION TITER OF RECOVERED ANTIGEN			
			AFTER WEEKS OF STORAGE		
METHANOL	ETHANOL	INITIAL	1	2	3
100	0	152	152	138	124
75	25	163	158	128	91
50	50	133	124	88	69
25	75	108	94	62	49
0	100	72	62	37	22

*Final alcohol cencentration 25 per cent by volume.

Adjustment of pH of the Infected Allantoic Fluid.—A pool was made of freshly harvested allantoic fluid infected with strain FM-1. This pool had an original pH of 8.25, and was divided into two equal portions. In one portion, the pH was adjusted to 7.0 with $NaH_2PO_4 \cdot H_2O$ solution, while the pH of the other portion remained at 8.25. Four aliquots from each of the two portions were transferred to flasks for treatment with methanol and with a 75 per cent methanol—25 per cent ethanol mixture. With either precipitating agent, this

adjustment of the pH did not result in differences in CCA titer of the final virus suspension or alter the stability of the titer upon storage at 4° C.

Determination of Optimal Methanol Concentrations.—Pools of freshly harvested allantoic fluid infected with influenza viruses and relatively free of interfering egg constituents were precipitated with varying concentrations of methanol in an effort to determine the concentration yielding the maximum recovery of virus.

Various increments of methanol were added by methods described above to the original infected allantoic fluid to obtain the desired final methanol concentrations.

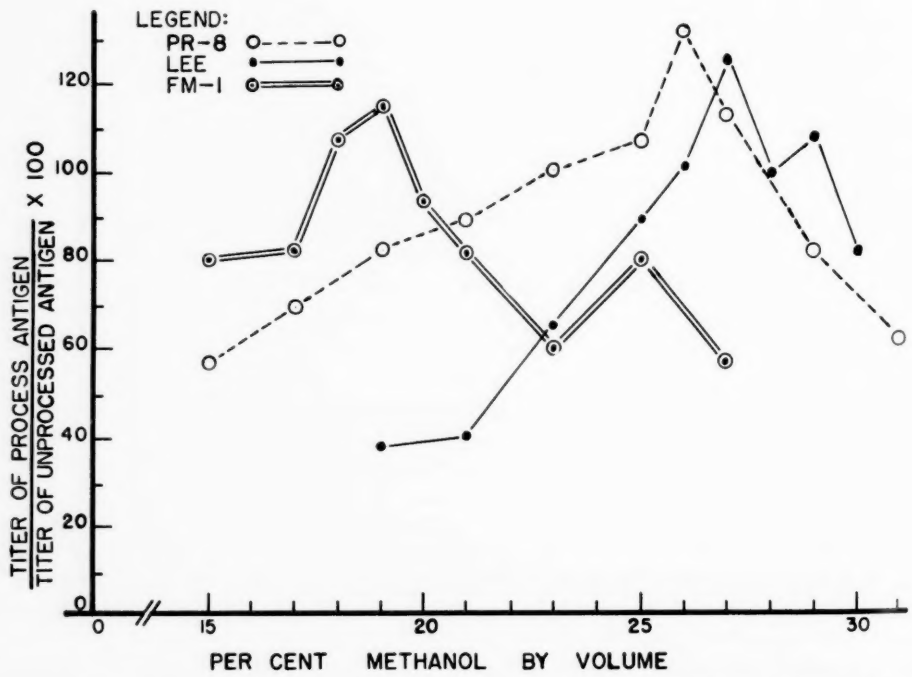

Fig. 1.—Optimal concentrations of methanol for precipitation of influenza antigens from allantoic fluids as measured by comparison of hemagglutination titers of processed and unprocessed antigen suspensions.

With strain FM-1, 2 per cent increments from 15 to 27 per cent were used in locating the optimal concentration range. Subsequently, 1 per cent increments from 17 to 21 per cent were used in obtaining a more definitive optimal concentration end point. It was determined that a concentration of 19 per cent gave the highest yield of virus, as illustrated in Fig. 1.

The value of 19 per cent was confirmed as the optimal concentration for precipitation of the FM-1 virus by repetition of the process in six additional pools of infected allantoic fluid. Optimal concentrations of methanol for the precipitation of strains LEE and PR-8 from infected allantoic fluid were similarly determined. The concentration ranges employed were 19 through 30

per cent for LEE, and 15 through 31 per cent for PR-8. The optimal methanol concentration for the LEE strain of virus was found to be 27 per cent. For the PR-8 strain, 0.5 per cent increments in the range of 25 through 27.5 per cent methanol gave good yields.*

These results were also confirmed in six additional pools of allantoic fluid infected with each virus.

Eluting and Suspending Buffers.—In an effort to determine the buffer for eluting and suspending the virus which afforded the greatest stability of the CCA titer of the resulting suspension, three phosphate buffer systems were considered.†

TABLE II. STABILITY OF HEMAGGLUTINATION TITER OF FM-1 STRAIN OF INFLUENZA VIRUS RECOVERED FROM PRECIPITATED ALLANTOIC FLUID (ORIGINAL TITER 247) AND SUSPENDED IN DIFFERING BUFFERS OF pH 7.0

BUFFER		PRECIPITATING AGENT* (PER CENT)		HEMAGGLUTINATION TITER OF RECOVERED ANTIGEN				
					AFTER WEEKS OF STORAGE			
SYSTEMS	MOLARITY	METHANOL	ETHANOL	INITIAL	1	2	3	52
Na_2HPO_4	0.1	100	0	304	294	231	201	128
and		75	25	315	256	215	163	—
KH_2PO_4	0.3	100	0	201	208	169	147	84
Na_2HPO_4		75	25	215	223	158	120	—
and	0.1	100	0	315	285	315	294	275
$NaH_2PO_4 \cdot H_2O$		75	25	285	285	256	176	—

*Final alcohol concentration 19 per cent by volume.

Each of the buffers employed yielded eluates containing adequate concentrations of virus, but upon storage at 4° C. the 0.1M and 0.3M sodium and potassium phosphate buffered suspensions formed precipitates with accompanying loss of CCA activity. Mono- and disodium phosphate buffered suspensions have remained clear and maintained a high CCA activity for over twelve months.

DISCUSSION

The optimal final concentrations of 27 and 26 per cent, respectively, for the precipitation of the LEE and PR-8 strains of influenza virus are at variance with the optimal concentrations which have been reported previously.[2, 3] Whether this variance is due to the use of differing seed strains or to antigenic variations in the strains incident to prolonged laboratory passage is not known. Further investigation will be required before the above concentrations can be accepted as optimum for precipitation of these strains in all laboratories. The FM-1 strain was optimally precipitated by a concentration of 19 per cent, suggesting that a considerable specificity exists for the methanol concentration

*Methanol concentrations of 19, 27, and 26 per cent, respectively, are now employed routinely in this laboratory for the precipitation of FM-1, LEE, and PR-8 virus antigens.

†0.1M sodium and potassium phosphate buffer. 8.7 Gm. anhydrous Na_2HPO_4 and 5.3 Gm. KH_2PO_4 per liter distilled water.

0.3M sodium and potassium phosphate buffer. 26.1 Gm. anhydrous Na_2HPO_4 and 15.9 Gm. KH_2PO_4 per liter distilled water.

0.1M mono- and disodium phosphate buffer. 8.7 Gm. anhydrous Na_2HPO_4 and 5.4 Gm. $NaH_2PO_4 \cdot H_2O$ per liter distilled water.

pH of all buffers adjusted to 7.00 ± 0.02 by use of electric pH meter.

which must be used in the precipitation of allantoic fluids infected with the several virus strains if maximum recovery of the virus is to be obtained.

In the processing of infected allantoic fluids to obtain virus suspensions for laboratory use, the greatest difficulty is not that of obtaining partially purified fluids of satisfactory virus concentration. Fluids of equal or greater purity can be prepared by several methods, although these for the most part are more complicated than the methanol precipitation and require special apparatus.[5, 6] The difficulty lies more in maintaining high levels of virus (CCA) activity upon storage. Neither the 0.1M nor the 0.3M sodium and potassium phosphate buffers proved satisfactory, there being a considerable loss of CCA titer within three weeks at 4° C. In contrast, the 0.1M mono- and disodium phosphate buffer has yielded suspensions of high CCA activity which have been maintained for months at 4° C.

Excessive quantities of extraneous nonviral substances in the infected allantoic fluid impairs the stability of the processed virus suspension. Some of the more common of these nonviral substances are bacteria, urease, lecithins, yolk material, membrane material, and embryonic cells. These seem to fix the virus in a spongy mass from which elution is difficult without carrying over a large amount of nonviral protein. Processed suspensions which are not clear rapidly lose their chick cell agglutinating activity.

SUMMARY

Studies were undertaken upon the preparation of purified and stable influenza virus for use in the serological diagnosis of influenza. Neither crude allantoic fluid or virus suspensions prepared following the methods of other workers had proved satisfactory because of the formation of precipitates with accompanying loss of virus activity upon storage.

Methanol was the most satisfactory precipitating agent. Each of three strains of influenza virus, PR-8, FM-1, and LEE, apparently have a characteristic of being precipitated optimally by methanol in final concentrations of 26 per cent for PR-8, 19 per cent for FM-1, and 27 per cent for LEE.

Alteration of the pH of crude allantoic fluid from 8.25 to 7.0 prior to precipitation with varying alcohol concentrations had no effect upon the yield of virus.

The buffer of choice for eluting and suspending the precipitated influenza virus was found to be a 0.1M mono- and disodium phosphate buffer, pH 7.0. Virus suspensions in this buffer, when stored at 4° C., have remained clear and maintained a high chick cell agglutinating activity for periods in excess of twelve months.

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NINHYDRIN-SCHIFF AND ALLOXAN-SCHIFF STAINING

A New Histochemical Staining Method for Protein

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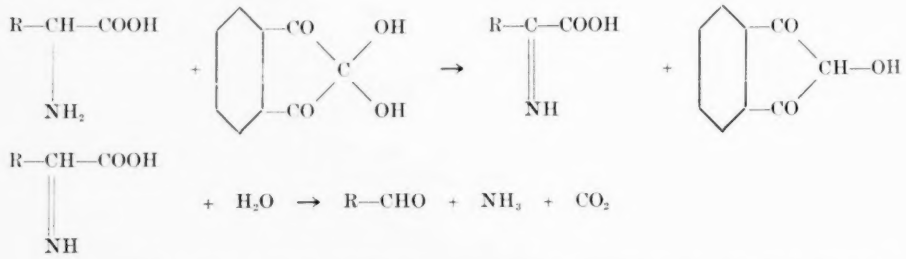
INTRODUCTION

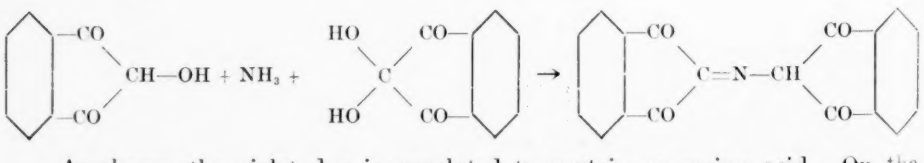
ALTHOUGH there are various methods for the histochemical identification of protein such as the ninhydrin reaction, diazo reaction, or Millon's reaction,[1] these are not advantageous because of poor coloration or damage to the tissues by the base or acid used.

The ninhydrin reaction, which is basically a biochemical reaction, has also been adopted cytochemically since the last century.[2] However, it has been applied unchanged in its entirety as a test-tube method, and this has caused diffusion artifacts in the sections.

In the course of its reaction ninhydrin is believed to split alpha-amino acids into three compounds, aldehyde, carbon dioxide, and ammonia during the first stage, as follows[3]:



The violet color, which indicates a positive reaction, is an end product resulting from condensation of ninhydrin, ammonia, and the reduction product of ninhydrin.



As shown, the violet dye is unrelated to protein or amino acid. On the other hand, amino acid is converted, as an ultimate product, into aldehyde derivatives (R-CHO) which remain in tissue and have no correlation with this dye. Moreover, the dye readily dissolves in water or alcohol and acts as an acid dye, and when a section of tissue is heated in ninhydrin solution, the dye can easily shift from one constituent of tissue and stain another.

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When we recall Berg's "sekundäre Färbung,"[2] this staining is not an unequivocal method and many investigators could not believe its validity, but they could not help using this method for demonstration of protein in tissues or cells.[5]

To avoid this error we paid attention to the demonstration of aldehyde residue which is assumed to be insoluble in tissues when the protein or polypeptide molecule is large. The aldehyde can be obtained as a dimedon-derivate, which shows that it is stable.[4] On the other hand, aldehyde combines with Schiff's reagent, as in Feulgen's technique[6] or Hotchkiss's P.A.S. staining,[7] where the aldehyde group is set free by pretreatment with hydrochloric acid or periodic acid, and subsequently exposed to fuchsinsulfurous acid. Thus, we inferred that when sections pretreated with ninhydrin are immersed in Schiff's reagent, protein or polypeptide in tissues will show a red color.

Besides ninhydrin, alloxan and isatin, as dicarbonyl compounds, are expected to act upon alpha-amino acid oxidatively. Our experiment was to utilize ninhydrin and alloxan, and we called this new method ninhydrin-Schiff staining (N.S. staining) and alloxan-Schiff staining (A.S. staining), respectively. Results with these two methods were clearly identical.

METHOD

1. Any routine fixative can be employed, but 10 per cent formol, Zenker's solution with and without 5 per cent acetic acid, or absolute alcohol are to be recommended.
2. Paraffin slides 5μ to 10μ in thickness are run through xylen twice and successively through absolute alcohol twice.
3. Incubate the slides at a temperature of 37° C. in 0.5 per cent ninhydrin or 1.0 per cent alloxan in absolute alcohol solution for five to twenty-four hours.
4. Wash gently under tap water for a few minutes.
5. Immerse slides in Schiff's reagent and proceed as in Feulgen's nucleal reaction.

Red or purple color indicates the distribution of protein or peptide.

RESULTS

Spot Test.—In order to confirm the validity of this reaction, a few samples of protein were selected for spot tests. Among these, ovalbumin was used in a highly purified form. It was dissolved in distilled water, smeared on the slide, and fixed in absolute alcohol before staining. The control which was immediately immersed in Schiff's reagent yielded almost no color, but sections pretreated by ninhydrin reacted with reduced fuchsin to give an intense red color.

The other protein samples showed almost the same results, but in evaluating these results it had to be considered that contaminated protein gave a red color on reacting with Schiff's reagent alone.

Digestion by Enzyme.—In order to lend validity to this method, an investigation was made by applying several enzymes to rule out protein and nonprotein substances from tissues.

Digestion by Pepsin: Paraffin sections of rat liver were immersed at 37° C. in a solution of crystalline pepsin (3 mg. per 100 ml. of 0.02N HCl). Digestion of tissues was noticeable first in intercellular substances and cell walls after five minutes, and subsequently the cytoplasmic granules and nucleus began to lose

their stainability in thirty to forty-five minutes. But controls which remained in 0.02N HCI at 37° C. for one hour showed no alteration of their color.

Digestion by Ribonuclease: A solution of ribonuclease (0.5 mg. per 100 ml. in pH 6.8 veronal buffer) was used for the removal of RNA. Hydrolysis was allowed to proceed for a period of one hour, but no evident differences were detected between enzyme digested sections and controls when stained with the N.S. method.

Digestion by Desoxyribonuclease: streptokinase-streptodornase* (S.K. 20,000: S.D. not less than 5,000 Christensen units) was dissolved in 20 ml. of physiological saline, and tissue sections were immersed in it for two and one-half hours at 37° C. In these specimens, when examined by Feulgen's reaction, evidence of complete nuclear dissolution was indicated by the disappearance of Feulgen positive desoxyribonucleic acid. But when stained by N.S.-staining, these sections showed red nuclei as in the controls.

In the course of this study, it was found that in smear preparations stained with Giemsa after digestion in this solution, the cell nucleus remained entirely unstained, while others showed pictures identical with those not digested by the enzyme. This limited observation will suggest that the substance stainable with Giemsa in the nucleus is indeed desoxyribonuleic acid and not protein.

From these studies, the conclusion could be drawn that this staining method is specific. Red or purple color obtained by this method indicates exclusively protein or peptide constituents in tissues.

Comparison of Results of the New Methods with Other Histochemical Reactions for Protein.—At present many methods are available for histochemical demonstration of protein. Most of these were devised so as to react with the reactive group in the protein side-chains and end-grouping of protein. For example, the phenolic side-chain of protein is responsible for the red color given by Millon's reagent, and side-chains of tyrosine or histidin are believed to be responsible for the rose color given by Pauly's diazo reaction. On the other hand, the color reaction brought about by aldehydes such as 2-dimethylamino-benzaldehyde (Ehrlich's reaction) is due to the presence of tryptophane.

The results with N.S. staining or A.S. staining were almost identical to that obtained by Millon's, Pauly's, or Ehrlich's reaction, all of which stained nuclei, fibers, and protoplasmic granules in liver cells.

Thus we infer that the N.S. and A.S. stainings will stain many types of protein in tissues. Chemically, this color reaction is due to the presence of free NH_2 and COOH in protein molecules. The superiority of N.S. staining and A.S. staining compared with the above-described methods consists in the intensive color and in the excellent preservation of tissue structure. Moreover, the stained preparations can be preserved for a long time. Nucleoli were stained distinctly red, and protoplasmic granules were delicately revealed in liver cells.

*Prepared at the Wellcome Research Laboratories of Burroughs Wellcome & Co., 9-11 East Forty-first Street, New York 17, N. Y.

SUMMARY

A new histochemical test for protein and peptide, based on the liberation aldehyde groups by ninhydrin or alloxan oxidation, and subsequent demonstration of aldehyde by reduced fuchsin, has been described. Experiments of enzymatic digestion and spot test showed the specificity of this reaction. It was compared with three present histochemical techniques for protein, and proved to be preferable in many respects.

This method, named A.S. or N.S. stain, gives a distinct color reaction, according to the distribution of protein in tissues.

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A STRETCHER-SCALE FOR THE ACCURATE WEIGHING OF BEDRIDDEN PATIENTS

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DAILY body weights often provide useful information concerning a patient's state of hydration and nutrition. This information is particularly important in cases presenting special problems in fluid and electrolyte therapy, since for practical purposes changes in daily body weights serve as a measure of changes in total body water. While records of fluid intake and output give the physician some idea of the fluid balance, their accuracy cannot always be relied upon. Furthermore, they do not include a measure of insensible water loss which may vary over a fairly wide range. Unfortunately, the conditions which most often indicate the determination of daily weights preclude the patient's standing on an ordinary platform scale. Scales have been developed for weighing the patient in the horizontal position,[1-3] but their size, complexity, or expense has prohibited their widespread use. It is the purpose of this paper to describe a simple, compact, mobile, and relatively inexpensive stretcher scale suitable for weighing bedfast patients.

CONSTRUCTION AND OPERATION

The stretcher scale is illustrated in Fig. 1. A platform type counter scale (Howe Scale Co., catalog No. 3301) was mounted on the undercarriage crossbeams of a wheel stretcher (Colson-Merriam Co., catalog No. 1N-10). The removable stretcher top was fitted with angle iron supports suspended from its undersurface so as to make contact with the scale platform at the time of weighing. At other times, the stretcher top is supported by four cams mounted on shafts at either end of the stretcher (Fig. 2). These cams are operated manually by handles attached to the cam shafts. This mechanism stabilizes the stretcher and protects the scale while the patient is getting on or off. Proper alignment of the stretcher top with the scale platform so as to allow free suspension is maintained by four conical pivots on the bottom of the support beams which fit into four conical depressions on the scale platform when the cams are lowered. A foam rubber mattress is held in place on the stretcher surface by means of a canvas cover which is laced to the stretcher top. The vertical dimensions of the apparatus are such that the top of this mattress is level with the top of the mattress of the standard hospital bed. One end of the stretcher top may be elevated to any desired height for comfort of the orthopneic patient When weights are being obtained, a draw-sheet is placed beneath the patient by which he may be

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Fig. 1.—The stretcher-scale.

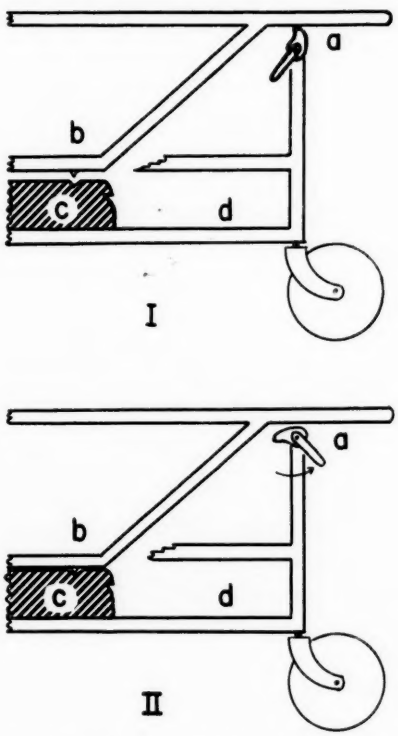

Fig. 2.—Diagram of the principle of suspension. In *I*, the stretcher top is supported by the cam (*a*) so that the truss (*b*) is out of contact with the scale platform (*c*) which is mounted on the under-carriage (*d*). In *II*, a turn of the handle and cam (*a*) has permitted the truss (*b*), and hence the stretcher top, to be supported entirely by the scale platform (*c*) so that weighing may be made.

moved gently to and from the stretcher. The weight of the draw-sheet must, of course, be included in the tare weight, which is then subtracted from the gross weight to obtain the net body weight.

ACCURACY AND REPRODUCIBILITY

The accuracy of the instrument and reproducibility of results have been checked periodically in two ways: (1) normal subjects have been weighed repeatedly over short periods of time, and (2) a known 1 kg. weight has been added to the litter while weighing patients to assess accuracy in measuring weight increments. Standard deviation in 20 weighings by the first method was ±0.006 kg. The mean weight increment obtained by adding a known 1 kilogram weight was 0.994 kg., with standard deviation of ±0.025 kg. in ten weighings. To obtain such accuracy, the center of gravity of the patient must be kept within the limits set by the width of the scale platform, about 12 inches. When this condition is met and the scales are manipulated with care and intelligence, it is possible to obtain the weights of bedridden patients within confidence limits of ±50 Gm. (±0.05 kg.). Such accuracy should be sufficient for most clinical and experimental purposes.

USES

The above-described apparatus provides a means for obtaining frequent and accurate body weights of bedridden patients who present problems in fluid balance and nutrition. It is especially useful in making possible exact weighings on patients undergoing certain balance studies which require such data. In this connection it has been used satisfactorily in approximately 300 weighings on the Metabolic Unit of the Hospital of the University of Pennsylvania. In addition it has been found particularly useful in measuring fluid exchanges during extracorporeal hemodialysis (water transfers between blood and dialyzing solution), as will be described in a later publication.

SUMMARY

A simple stretcher-scale for weighing of bedfast patients has been described. Its design, construction, accuracy and clinical application have been discussed.

The authors are indebted to Mr. Robert Mather of the Physiology Shop, University of Pennsylvania, for assistance in the design of this apparatus and for its construction.

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EXCLUSION OF AIR-BORNE CONTAMINATION IN FLAME PHOTOMETRY

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THE usefulness of the flame photometer for rapid and accurate analyses of sodium and potassium in biological fluids has been demonstrated repeatedly.[1-6] Two types of instruments have been used, those having a closed air system utilizing compressed air and those depending on room air to supply oxygen to the flame. The efficiency of the latter system is greatly dependent on a dust-, smoke-, and draft-free atmosphere.[7, 8]

In the authors' laboratory it was not possible to avoid heavy contamination of the room air by dust from adjacent new construction. The design of our instrument* allowed air to reach the flame directly by convection, sweeping over large areas particularly susceptible to dust accumulation. Due to increasing contamination of the room air, it became progressively difficult to obtain accurate analyses, particularly of potassium. This problem has been avoided by the use of a separate air-conditioned room or by employing a closed air system.[8, 9] Olney and James[7] have recently described an air-cleaning apparatus, which decontaminates the room air reaching the flame by means of a high-voltage dust precipitator. The present paper describes a simple device for converting a flame photometer, which receives its air supply from the room air by convention, into one to which filtered or selected air may be supplied.

The flame of the photometer used by the authors* receives its air from three sources, the main portion entering through vents at the base of the burner. This will be referred to as the internal air supply. In addition, room air has free access to the flame at the top of the burner. This source of air will be referred to as the "external" air supply. Thirdly, air enters the burner along with the discharge from the atomizer. Contamination from this last source may be controlled since it is supplied directly from a source of compressed air.

In our instrument contamination by dust entering the base of the burner was eliminated by wrapping the gap between the burner and the proximal end of the chamber of the atomizer with aluminum foil. It was found necessary to supplement the air reaching the burner with air from the compressed air line, which supplied the atomizer. The air was led from a T-tube into the chamber between the base of the burner and the vent of the atomizer by means of a small, bent glass tube. A short length of capillary tubing was used to reduce the pressure. All the openings around the tube supplying the air were

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tightly sealed with aluminum foil. While determining the optimal supply of air to this source, a water manometer was inserted in the line. When admitting air to the base of the burner through a tubing of approximately 3 mm. internal diameter, an air pressure of 35 to 50 mm. of water was found sufficient. This pressure can alternatively be controlled by regulation of the reducing valve which controls the supply of air to the atomizer.

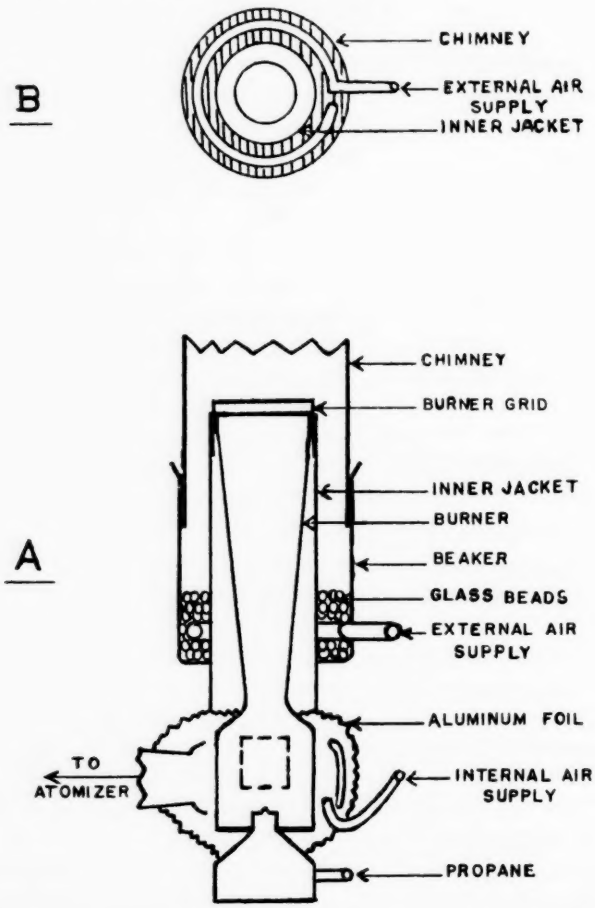

Fig. 1.—*A*, Longitudinal section of burner and chimney with devices to exclude room air. *B*, Cross section at level of external air supply.

It was found that minor contamination by dust could be controlled by this device alone. However, a further modification of the instrument was necessary for satisfactory operation in the face of increasing contamination by air. A cylindrical chamber was designed to fit tightly around the burner proper. Details of construction are shown in Fig. 1. The inner jacket surrounding the burner was cut from copper pipe of about 30 mm. outer diameter and splayed at the upper end to accommodate the downward extension of the burner grid. A 400-ml. stainless steel beaker about 80 mm. in diameter was

used as the outside jacket. A 30-mm. opening for the inner jacket was cut out of the bottom of the beaker, and the inner jacket was soldered to the steel beaker at this point. A 7-mm. piece of copper tubing with a sealed end was inserted through an opening about 0.5 cm. above the bottom of the beaker to provide an inlet for air. This was bent in a circle so as to lie just above the bottom of the beaker. A series of small perforations was made on the under side of the circular portion of this tubing. In order to insure an even flow of air, a layer, 1 inch in depth, of glass beads about 4 mm. in diameter was placed as a baffle overlying the circle of tubing. To provide an adequate supply of air to the flame, it was found preferable to connect the air inlet directly to the main compressed air line. The pressure in this line was controlled by means of a tunnel clamp and was gauged by a water manometer. Contamination by oil or dirt transmitted through the compressed air line was avoided by placing a felt filter, 2 inches in length and 1 inch in diameter, in the main air line. The flame obtained with this modified apparatus was, if anything, superior to that produced by a free supply of air. The entire adaptation may be inexpensively and easily constructed in a short time by a competent mechanic. As the unit is independent of the burner, the grid, and the chimney, the parts may readily be removed for cleaning or repair. With slight modification, the device could be applied to any flame photometer employing an open air supply, without change in the basic parts. The operating characteristics of the instrument are not otherwise changed. No alterations are necessary in the size of the windows which admit light to the photoelectric cells. The hazard of explosion of gas accumulated within the apparatus is diminished, since the burner is essentially isolated, and in this device no electrostatic precipitation of dust is involved.

The structural alterations have resulted in a somewhat higher thermal equilibrium, which is reached after the instrument has been warmed up for thirty to forty-five minutes.

These alterations have made the instrument entirely satisfactory for routine clinical and research analyses, in spite of difficult environmental conditions. It has been possible to keep within a mean error for sodium analyses of 0.4 per cent and for potassium analyses of 1.6 per cent in recoveries of known solutions. Potentiometer readings for standard solutions have remained stable for hours after the initial period of warming up. Erratic fluctuations in the readings of the galvanometer have been almost entirely eliminated.

SUMMARY

A device is described which eliminates contamination by dust in flame photometers utilizing an open air supply.

The authors are indebted to Dorothy W. Sears, Jr., and to Edward Merrill for valuable technical assistance.

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THE QUANTITATIVE DETERMINATION OF PLASMA HEMOGLOBIN BY THE BENZIDINE REACTION

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FEW methods for the determination of plasma hemoglobin are reported in the literature. Flink and Watson[1] have published a colorimetric method based on the formation of pyridine hemochromogen. Attempts to apply this method have failed in this laboratory because the occurrence of varying degrees of turbidity, even in duplicate samples, has made photometric comparison impossible.

We have discarded the benzidine dihydrochloride method of Karr and Chornock.[2] The suggested artificial standards were too low when compared with standards prepared from blood of known hemoglobin concentration as determined by the oxygen capacity method. Furthermore, using these known hemoglobin solutions, the results were too erratic to establish a standard curve.

Ham[3] has proposed a modification of the Bing and Baker[4] microdetermination of hemoglobin in whole blood, and it is a further modification of this benzidine reaction which is being reported here.

MATERIALS AND METHODS

Reagents and Apparatus.—(a) Acetic acid, glacial; (b) acetic acid, 20 per cent; (c) benzidine, 2 per cent. Benzidine base is purified according to the method of McFarlane and Hamilton.[5] The final recrystallization from 95 per cent ethyl alcohol is repeated three times or until the liquor remains crystal clear. Two grams of this purified product are dissolved in 20 ml. of glacial acetic acid with the aid of gentle heat, further diluted to 100 ml. with water, treated with 1 Gm. of decolorizing charcoal, and filtered. The filtrate should be crystal clear. (d) Hydrogen peroxide 1.5 per cent freshly prepared from 30 per cent H_2O_2.

(e) Standard hemoglobin solutions are prepared by appropriate dilution of blood, the exact hemoglobin concentration of which has been determined by the oxygen capacity method. Such standards are stable up to thirty days when kept in a refrigerator.

(f) Glassware: All glassware must be scrupulously clean, since many contaminating substances will cause color development.

Procedure.—(a) Plasma is diluted to contain less than 10 mg. per 100 ml. of hemoglobin. 0.5 ml. is pipetted into a 100 ml. volumetric flask.

(b) Two milliliters of benzidine reagent is added to the flask and the contents are well mixed.

(c) One milliliter of 1.5 per cent H_2O_2 is pipetted into the flask which is agitated and permitted to set at room temperature for one hour. The initial deep blue color gradually turns brown and finally turns to a stable purple.

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(d) After incubation the flask is filled to the 100 ml. mark with 20 per cent acetic acid, mixed, and permitted to set for thirty minutes.

(e) The optical density of the sample is determined in a Beckman Model B photoelectric colorimeter at a wave length of 490 mμ against a reagent blank at 0.

(f) A standard hemoglobin curve in the range of 1 to 10 mg. per cent is determined with each series of samples.

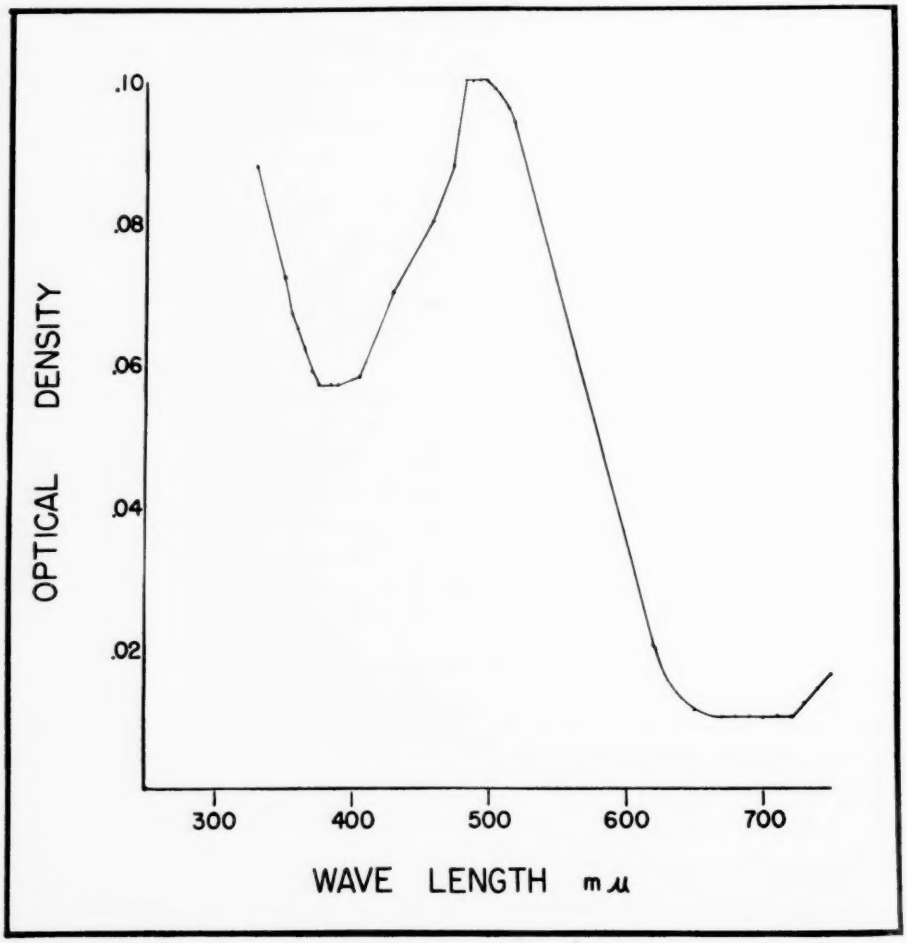

Fig. 1.—Absorption spectrum.

(g) Two plasma recoveries with different amounts of added hemoglobin are run with each series of determinations. For each dilution of plasma, separate recoveries must be run so that the final dilution of plasma used for the recovery is the same as for the unknown. Table I illustrates the procedure for two different plasma dilutions.

Calculation.—Hemoglobin concentration mg. per cent $= \frac{C}{R} D$

C = concentration from standard curve.
R = average of two recoveries.
D = plasma dilution.

RESULTS AND DISCUSSION

The use of benzidine purified as suggested, and the choice of a final volume of dilution of 100 ml. rather than 25 ml. as in the Bing and Baker method,[4] minimize the blank reading contributed by the reagents.

The absorption spectrum for the final color is plotted in Fig. 1. A plateau is reached between 485 and 500 mμ. Using 1.5 per cent H_2O_2, the color is stable up to twenty-four hours.

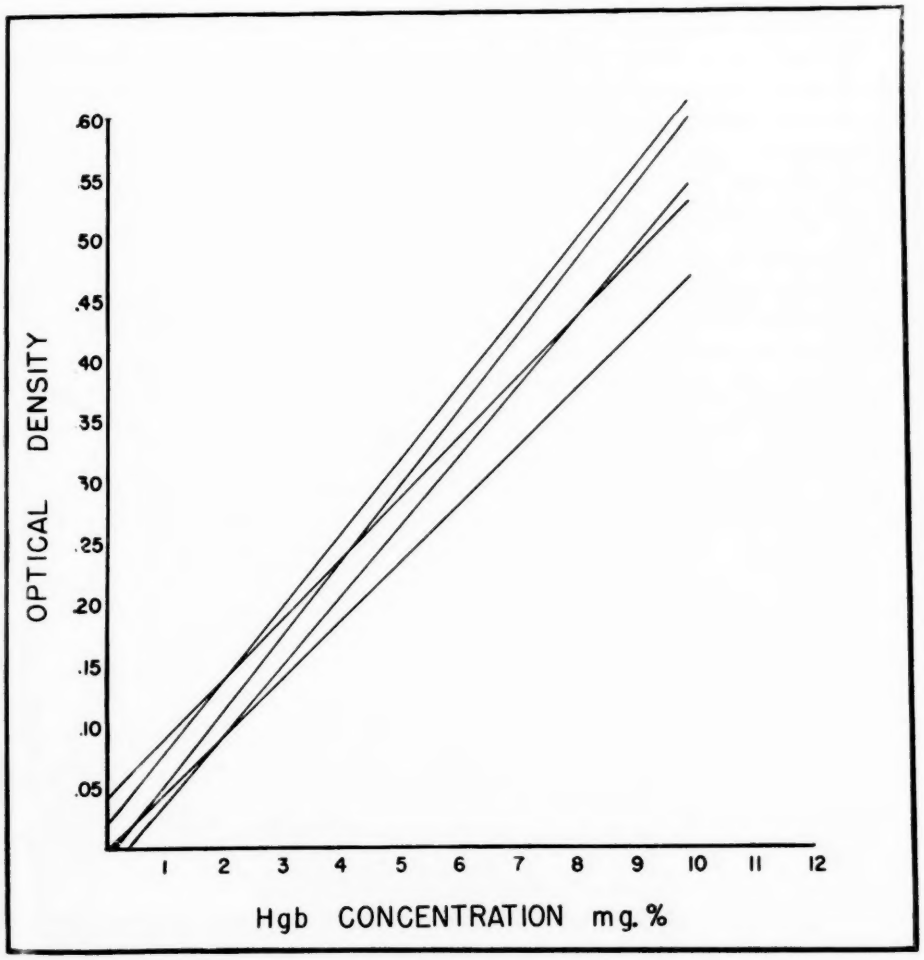

Fig. 2.—Variations in standard curves.

The standard curve obeys Beer's law in the range of 1 to 10 mg. per 100 ml. However, the slope of the line varies slightly with each freshly prepared batch of benzidine reagent (Fig. 2). Therefore, a standard curve must be run with each series of determinations. This random variation is not a function of instability of the hemoglobin standards.

TABLE I. SCHEME FOR RECOVERY OF HEMOGLOBIN FROM PLASMA AT DIFFERENT PLASMA DILUTIONS (1:2 AND 1:11)

ML. PLASMA	ML. H_2O ADDED FOR DILUTION	ML. STD. SOLUTION ADDED	CONCENTRATION STANDARD SOLUTION (MG. %)	FINAL PLASMA DILUTION	DILUTION OF STD. SOLUTION	THEORETICAL RECOVERY (MG. %)
1	1	0	—	1:2		
1	0	1	14.0	1:2	1:2	7.0
1	0	1	4.3	1:2	1:2	2.15
1	10	0	—	1:11		
1	0	10	7.6	1:11	10:11	6.9
1	0	10	5.1	1:11	10:11	4.6

Low plasma recovery values have been uniformly observed, suggesting that plasma interferes with the benzidine reaction. Heating at 56° C. for one hour does not alter the degree of interference. The recovery values range from 40 to 70 per cent (average 61.2 per cent) with plasma dilutions up to 1:25 and over 85 per cent with high plasma dilutions (1:100) (Table II). However, there is no consistent relationship between plasma dilution and per cent recovery within the broad ranges mentioned.

In spite of this variability, it has been consistently observed that simultaneous recovery of hemoglobin at any given level of plasma dilution will yield values which differ by a maximum of only 6.6 per cent (average 3.2 per cent) (Table II). This agreement permits the use of the recovery value in the calculation of hemoglobin concentration.

TABLE II. RECOVERY OF HEMOGLOBIN FROM PLASMA

PLASMA DILUTION	THEORETICAL RECOVERY MG. %	ACTUAL RECOVERY	PER CENT RECOVERY	DIFFERENCE
1:2	7.00	3.02	43.1	1.5
	4.37	1.82	41.6	
1:2	5.60	3.46	61.8	0
	4.20	2.60	61.8	
1:2	5.18	3.15	60.7	1.8
	2.59	1.62	62.5	
1:2	5.18	2.88	55.6	3.6
	3.89	2.30	59.2	
1:2	5.18	3.45	66.6	2.3
	3.89	2.50	64.3	
1:2	5.18	3.50	67.5	3.2
	3.89	2.50	64.3	
1:2	5.18	3.08	59.4	1.0
	3.89	2.35	60.4	
1:11	5.09	3.05	60.0	1.8
	7.57	4.68	61.8	
1:11	7.06	4.29	60.7	3.0
	4.71	2.72	57.7	
1:11	7.06	5.35	75.8	5.0
	4.70	3.33	70.8	
1:17	7.32	5.05	69.0	6.5
	4.88	3.05	62.5	
1:100	7.77	7.21	93.0	5.8
	5.18	5.11	98.8	
1:200	7.77	6.45	83.0	6.6
	5.18	4.65	89.6	
			Average	3.2

The benzidine methods found in the literature have utilized visual colorimetry, and the variability in the standard curves may not have been recognized. In addition, no data have been presented regarding plasma recoveries.

It is felt that these limitations of the benzidine reaction must be appreciated for any quantitative application of the reaction to plasma. With the precautions noted, the accuracy is limited to the variation in recoveries. However, the sensitivity is great, as indicated by the standard curve range of 1 to 10 mg. per cent.

SUMMARY

A method for determination of plasma hemoglobin content based upon the benzidine reaction has been presented.

The method is sensitive to change in hemoglobin concentration between 1 and 10 mg. per cent.

Variability of the standard curve and incomplete recovery of hemoglobin added to plasma have been observed. The latter limits the accuracy of the method.

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THE DETERMINATION OF CREATININE IN PLASMA AND URINE IN THE PRESENCE OF LARGE AMOUNTS OF PHENOL RED

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IT IS often desirable to study the simultaneous clearance or renal extraction of creatinine and phenol red. In order to carry out these experiments at high plasma and urine concentrations of the dye, it was necessary to devise a way to eliminate its color which interferes in the conventional alkaline picrate method for determining creatinine.[1] The procedure presented herein is based on the fact that the phenol red present in urine or plasma filtrates can be converted to the colorless base by boiling with zinc dust[2] and that the creatinine content of samples thus treated remains unchanged.

PROCEDURE

Creatinine is determined according to a modification of the Folin-Wu[1] method.

Reagents.—

1. Sulfuric acid, 0.67N;
2. Sodium tungstate, 10 per cent, aqueous;
3. Picric acid, saturated, aqueous;
4. Sodium hydroxide, 10 per cent, aqueous;
5. Zinc dust;
6. Alkaline picrate solution: Mix just before using: 5 volumes picric acid reagent, 6 volumes distilled water, and 1 volume sodium hydroxide.

Preparation of Plasma Filtrate.—

Add while mixing well: 1 volume of plasma, 8 or 18 volumes distilled water, ½ volume sulfuric acid, and ½ volume sodium tungstate. Let stand for ten minutes. Filter.

Analysis.—

1. To 10 ml. aliquots of filtrate or dilute urine in graduated test tubes add approximately 1 Gm. of zinc dust. Place tubes in a boiling water bath for twenty minutes.

2. Cool to room temperature, adjust volume, and filter. (If decolorization is not complete, the zinc treatment should be repeated.)

3. Pipette one volume of sample into a colorimeter tube. Add two volumes of alkaline picrate solution, mixing well.

4. After exactly fifteen minutes, read colorimetrically at a wave length of 515 mμ. Compare readings with a previously prepared standard curve that was not subjected to the zinc dust treatment.

RESULTS

The recoveries of creatinine from plasma containing up to 20 mg. per 100 ml. of phenol red were 95 per cent or better. Most of these recovery data were obtained by injecting creatinine subcutaneously into dogs, withdrawing samples of blood after twenty to thirty minutes, and then preparing duplicate

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TABLE I. A COMPARISON OF THE PLASMA CREATININE LEVELS OBTAINED WITH AND WITHOUT THE ADDITION OF 20 MG. PER 100 ML. PHENOL RED, AS DETERMINED FOLLOWING ZINC TREATMENT OF THE SAMPLES

MG./100 ML. WITHOUT PHENOL RED	MG./100 ML. WITH PHENOL RED	% RECOVERY
18.0	17.6	97.8
20.4	20.4	100.0
24.0	23.0	95.8
16.8	16.4	97.6
18.8	18.0	95.7
16.4	16.8	102.4
14.2	14.8	104.2
19.8	20.4	103.0
11.6	11.6	100.0
17.4	17.2	98.9
	Av.	99.5

filtrates of the plasma with and without the addition thereto of phenol red. Table I summarizes results obtained when phenol red was added to plasma to give a concentration of 20 mg. per 100 ml.

Urinary recoveries of creatinine in the presence of varying concentrations of phenol red are given in Table II. To obtain these data urine samples were divided into five portions. An equal amount of creatinine was added to each portion, as well as increasing amounts of phenol red. In every case the sample

TABLE II. THE EFFECT OF INCREASING AMOUNTS OF PHENOL RED ON CREATININE RECOVERY FROM DOG URINE AS DETERMINED BY THE MODIFIED PROCEDURE DESCRIBED HEREIN

MG./100 ML. PHENOL RED	COLORIMETER READINGS	MG./100 ML. CREATININE	% RECOVERY
0	64^3	5.58	
	65^1		
1	63^2	5.80	103.9
	64		
5	63^3	5.80	103.9
	64		
10	64^2	5.70	102.2
	64		
15	64^3	5.58	100.0
	65^1		
0	38^3	13.20	
	38^3		
1	38^3	13.40	101.5
	38^1		
5	38^3	13.20	100.0
	39		
10	38^2	13.40	101.5
	38^2		
15	37	13.80	104.5
	37^2		
0	19^3	25.00	100.0
	19^3		
1	19^2	25.22	100.9
	19^2		
5	19^2	25.22	100.9
	19^2		
10	19^3	25.60	102.4
	19		
15	19^2	25.60	102.4
	19		

to which no phenol red had been added was run through the usual creatinine procedure in order that its value could be used for a reference standard. All other samples were subjected to the zinc dust treatment before being analyzed for creatinine.

More difficulty has been encountered in this laboratory in reducing phenol red added in vitro than in reducing equivalent amounts of the dye in plasma obtained from animals that have received the compound. For example, filtrates of in vivo plasma samples containing as much as 36 mg. per 100 ml. of phenol red have been decolorized with no trouble, while it is usually difficult to remove the color from similar filtrates prepared from plasma to which more than 20 mg. per 100 ml. of the dye has been added. However, when decolorization is not complete following a single zinc reduction, a second zinc reduction invariably has produced a colorless sample.

TABLE III. THE EFFECT OF ONE AND TWO ZINC REDUCTIONS ON ENDOGENOUS CREATININE RECOVERIES FROM URINE CONTAINING 20 MG. PHENOL RED PER 100 ML.

MG./ML. CREATININE	MG./ML. CREATININE WITH PHENOL RED	% RECOVERY
	One Zinc Reduction	
1.975	1.925	97.5
1.575	1.625	103.2
1.925	1.975	102.6
2.70	2.65	98.2
2.85	2.85	100.0
2.325	2.25	96.8
	Av.	99.7
	Two Zinc Reductions	
4.30	4.25	98.8
3.475	3.50	99.3
2.10	2.25	107.1
2.85	2.65	93.0
2.90	2.675	92.2
2.25	2.125	94.4
	Av.	97.5

Creatinine recoveries from samples treated a second time with zinc are satisfactory, although the additional handling apparently increases the experimental error as shown in Table III. To evaluate this point, urine from twelve dogs that had been given creatinine was collected and was assayed for that compound by the alkaline picrate method. Sufficient phenol red then was added to every sample to give a concentration of 20 mg. of dye per 100 ml. of urine, and portions of each were decolorized by the zinc dust treatment. One-half of these aliquots, picked at random, were treated with zinc dust a second time. Creatinine determinations were performed in order that values obtained following a single and repeated zinc reductions could be compared. This is a typical experiment since a greater spread of recovery data occurred following two zinc reductions. Fortunately, the error involved herein is of little consequence since it seldom is necessary to repeat the decolorization process in the course of the experiment.

SUMMARY

A method for reducing phenol red in order to eliminate its interference in creatinine determinations has been described. This procedure depends on the fact that boiling samples with zinc dust discharges the phenol red color and thus makes it possible to apply a conventional alkaline picrate creatinine procedure. Creatinine recoveries from both plasma and urine containing the dye have been presented.

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LABORATORY FACTORS INFLUENCING THE DETERMINATION OF PLASMA VOLUME USING HUMAN ALBUMIN TAGGED WITH I^{131}

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WITH the availability of serum albumin firmly tagged with radioactive iodine, the determination of plasma volume in both animals and man has become technically relatively rapid and simple. Fine and Seligman[1] used this material to determine the plasma volume in experimental animals, and more recently Storaasli and associates[2] employed human albumin tagged with I^{131} to determine plasma volume in human subjects. The radioactive-labeled albumin offers a good tool for the determination of plasma volume because the albumin mixes well with the blood and leaves the vascular tree slowly; the isotope can be detected easily with the Geiger-Müller or scintillation counter; small amounts of hemolysis do not interfere in the determination of the radioactivity; and the amount of radioactive material given is too small to produce any untoward effects in the subject.

During the course of another study, it was desired to measure with a high degree of precision changes in plasma volume in experimental animals and man. Since some of the laboratory procedures involved in this determination were not completely in control, it was decided to investigate a group of these factors which might influence the plasma volume as determined by using albumin tagged with I^{131}.

METHODS

Human albumin tagged with radioactive iodine was obtained from commercial sources.* Measurements of activity of I^{131} (beta particles) were made on a Berkeley Decimal scaler with a Geiger-Müller tube, mounted vertically in a shielded lead container. All samples to be counted were pipetted (1 ml.) into porcelain milk evaporating dishes, 43 mm. in diameter, which fitted into the sample holder of the counting chamber. One milliliter of plasma just covered the bottom of the counting dish. All the samples of plasma, standards, and tests were allowed to dry at room temperature overnight before their activity was estimated.

Solutions used for most of the present work were the same as the standards in the determination of plasma volume as described in the method of Storaasli and associates.[2] These were prepared by diluting 1 ml. of iodinated human albumin to 100 ml. with either normal human plasma or reconstituted lyophilized human plasma. Other modifications in technique will be discussed under the various headings in the results.

RESULTS

Variation in Pipetting Samples.—As a source of radioactive material, human plasma was fortified with human albumin containing I^{131} so that the final

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solution gave about 750 counts per minute per milliliter. Four different 1 ml. Exax Blue Line pipettes were filled with the radioactive solution and pipetted, in duplicate, into selected counting dishes. It was found that the variation between duplicates ran between 0.6 to 2.3 per cent, with about 1 per cent being the mean variation. Since the counting of any radioactive sample was normally carried only sufficiently long to have the counting error within 1 per cent, the variation due to pipetting is within this range (Table I).

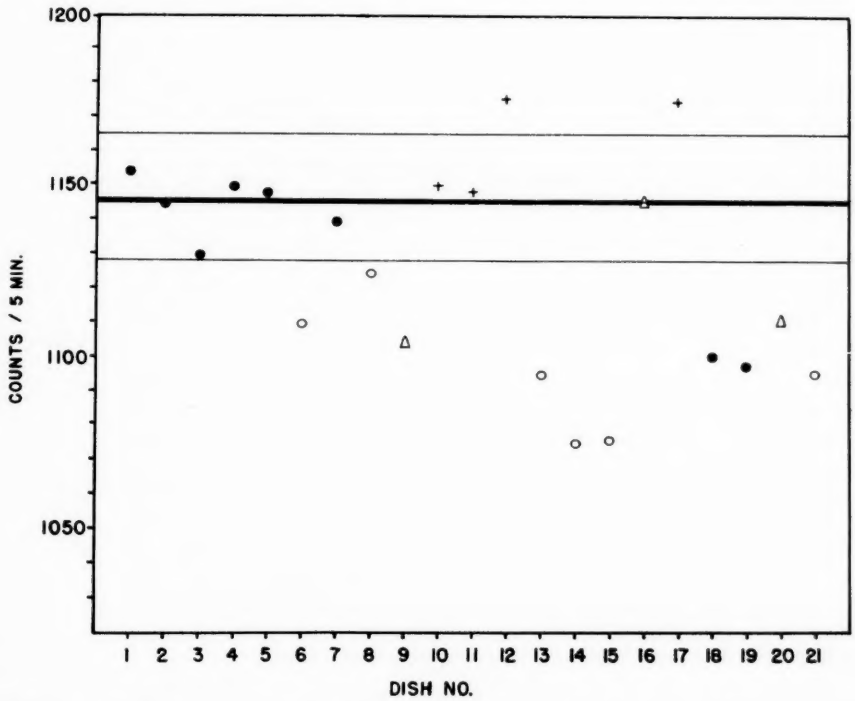

Fig. 1.—The effect of variation in dry protein film on the counting. The solid circles represent dishes which had dried films of protein which were flat and even. The open circles represent dishes which had thin films in the center. The triangles represent dishes in which the film was thick on one edge. The crosses designate dishes which had thick film in the center. The heavy line is the mean derived from seven selected dishes in which the protein film was even. The light lines represent the two σ limits.

Variation in Sample Holders.—One of the basic assumptions in the use of the Geiger-Müller counter for the determination of plasma volume by means of albumin tagged with I^{131} is that the geometry of the system does not change between samples and the standard. This is somewhat fixed by having the Geiger-Müller tube permanently mounted in a shielded container in which the samples can be placed only in a definite position in relation to the tube. In the present work, the sample dishes were selected by visual observation and only those possessing flat bottoms were chosen. But after the samples were dried in the dishes it was noted that in many cases the film of dried plasma was not even and flat. The effect of variation in the film surface of the dried plasma on count is given in Fig. 1. By using carefully selected dishes in which the inside bot-

TABLE I. PIPETTING VARIATION

EXAX 1 ML. PIPETTE	COUNTS/5 MIN.	DIFF. IN DUPLICATES
No. 1	3,705	58
	3,690	
2	3,745	87
	3,658	
3	3,787	24
	3,811	
4	3,767	164
	3,603	
	Mean 3,740	

tom surface was flat, little variation was found between samples. On the other hand, dishes which had depressions in the center producing thick layers of plasma in the center gave high counts (dishes No. 10, 11, 12, and 17). Those which had slight elevation in the center produced films which were thinner in this area and usually gave lower counts than the flat dishes (No. 6, 8, 13, 14, 15, and 21). The dishes which had pitted areas in the center usually gave high readings.

An attempt to produce more uniform counting surfaces was made by lining the individual dishes with filter paper discs which just covered the bottom. It was thought that the plasma could coat the paper and in this manner eliminate any effect of variation in the bottom of the dish. The results are given in Fig. 2. The selected dishes gave counts which had a small variation whether the filter paper was added or not, while dishes with pitted areas yielded higher values in spite of the addition of the filter paper liners.

Some of the samples of plasma after drying had a tendency to flake. Unless the particles redistribute in a definite manner, there is little effect on the count. Flaking rarely occurred in the standards. Attempts were made to eliminate this by adding one drop of a 10 per cent solution of carbitol acetate to the dish before pipetting the 1 ml. of plasma. The carbitol acetate acted as a plasticizer to prevent the flaking.

The present work would indicate that to get reproducible results it is necessary to obtain counting dishes which are perfectly flat on the bottom and to dry the plasma samples on a surface which is level. The latter precaution will eliminate the difference in surface thickness of dried plasma film caused by flowing of the plasma toward the lower side of the dish.

Distance Between the Geiger-Müller Tube and Sample.—The number of counts one obtains per unit of time from a given sample depends on both the activity of the sample and the distance the source of beta radiation is from the Geiger-Müller tube, providing the tube is operating on its voltage plateau. The activity of a sample, as measured by the Geiger-Müller counter, varies inversely as the square of the distance between the sample and the tube. The advantage of having the tube close to the sample is that the time necessary to obtain a statistically significant number of counts is short. On the other

hand, the closer the sample is to the tube the more unevenness of the sample layer influences the count. Table II gives that data obtained by counting the same seven sample dishes to which 1 ml. of plasma containing radioactive iodine was added at different distances from the Geiger-Müller tube. The time for counting was varied, so that approximately the same number of counts was obtained at each level. Thus, one can see that putting the sample on a lower shelf markedly increases the time to record about the same number of disintegrations. This increase in time can be very significant if one has many samples to count, such as occurs in carrying out disappearance curves or multiple plasma volumes. Since variation in counting is about the same on any one shelf, it appears to be advantageous to use the shortest practical distance between the tube and the sample so that the counting time becomes short.

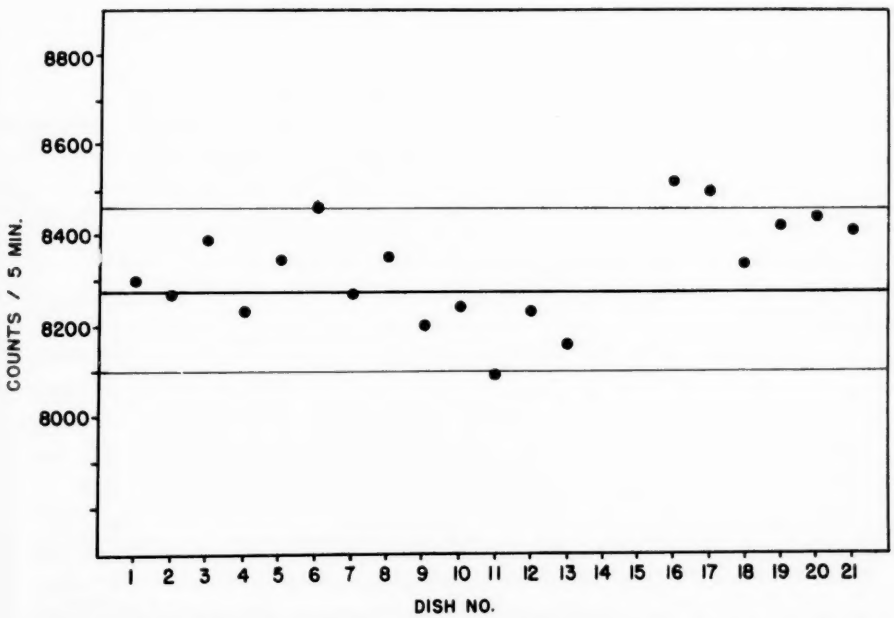

Fig. 2.—The effect of irregularities in sample dishes on counting variation. The heavy dark line represents the mean value for thirteen selected counting dishes. The light line is the two σ limits. Dishes number one to thirteen were specially selected for evenness. Dishes fourteen to nineteen had various pitted areas. All dishes had a filter paper liner.

Variation in Protein Content of the Standard.—In all determinations of plasma volume using albumin tagged with radioactive iodine, a portion of the material injected (usually 1 ml.) is diluted with normal plasma to either 50 or 100 milliliters. One milliliter of this diluted material is used as the standard for evaluating the activity of the plasma obtained from the patient. The use of such a standard eliminates the necessity of calculating the decay of the I^{131} and assumes that all corrections for self-absorption are automatically eliminated. The assumption is made that factors present in the serum of the patient which cause self-absorption are the same as those present in the standard. To test

TABLE II. EFFECT OF SHELF HEIGHT ON COUNTING PRECISION

SHELF NO.	DISTANCE FROM GM. TUBE (MM.)	NUMBER OF SAMPLES COUNTED	TIME OF COUNTING	TOTAL NO. OF COUNTS PER SAMPLE
1	15	7	1 min.	13472 ± 197
2	32	7	2 min.	13739 ± 187
3	47	7	4 min.	14018 ± 195

this, samples of plasma used in making the standard were diluted with saline to reduce the protein content to various levels, and then this diluted plasma was used in making up the standards. If plasma was diluted in such a manner that the protein content fell about 18 per cent, then there was an increase in the number of counts obtained. On decreasing the protein content to 50 per cent, there was a further increase in the number of counts given by the sample. Using data obtained previously on the activity of plasma of patients who were given the same lot of tagged albumin for the determination of plasma volume, and using the above two standards, it was found that decreasing the plasma protein content of the standard about 18 per cent increased the plasma volume of the patient by about 2.8 per cent in one series and 4.2 per cent in the other. When the plasma protein of the standard was further reduced to 50 per cent, the calculated plasma volume of the patients increased 5 to 12 per cent, respectively (Table III). These data indicate that the difference in protein content of the plasma of the patients and that of the standard might influence the value one obtains for the plasma volume. The difference in protein content would cause a difference in self-absorption between the standard and the test plasma. Since the plasma used for making the standards was relatively uniform,* with a protein level of about 6 Gm. per 100 ml., the major variation would occur in the protein content of the plasma obtained from the patients. The expected changes would in general be toward lower protein levels which would in effect cause the estimated plasma volume to be lower than the actual value. It is quite easy to reduce the error caused by difference in protein content of the standard and test samples by first determining the protein content of these two plasmas by one of the rapid methods (quantitative biuret or specific gravity procedures). Then dilute the normal plasma which is to be used as the standard to the same protein content as the

TABLE III. EFFECT OF PROTEIN CONCENTRATION OF THE STANDARD ON THE PLASMA VOLUME

STANDARD NO.	PROTEIN CON. GM./100 ML.	COUNTS/MIN.	PLASMA VOLUME*	DIFF. FROM CONTROL	
				ML.	%
1	6.25	1,165	3,320	--	--
2	5.18	1,197	3,412	92	2.8
3	3.13	1,224	3,489	169	5.1
1	6.75	1,729	2,945†	--	--
2	5.62	1,798	3,068	123	4.18
3	3.87	1,936	3,304	359	12.19

*Figure calculated on the data obtained from EC. carried out at this day.
†Plasma volume from the data obtained from RR. performed at this time.

*Blood bank discards and reconstituted lyophilized plasma.

TABLE IV. WASHING PIPETTES AND COUNTING DISHES*

PIPETTES	COUNTS/MIN.	
Control	25.7	Pipette not used for I^{131}
Washed 10 min. in pipette washer, suction dried	22.2	
10 min. in cleaning solution and 10 min. in pipette washer	23.7	
Washed by hand with tap water and distilled water, suction dried	25.5	
Syringe filled with I^{131} solution of albumin and washed with detergent and dried in routine manner	26.0	

*All pipettes and other equipment were filled with standard solution of I^{131}-tagged albumin in plasma, then washed in various manners and filled with distilled water and the latter checked for activity.

test plasma (within 0.5 Gm. per 100 ml.). In this manner the difference due to self-absorption between the standard and test plasma is negated.

Effect of Washing Glassware on the Removal of Radioactive Contamination.—In all measurements of radioactivity the possibility of contamination with materials which yield counts need to be considered. The fact that I^{131}-tagged albumin might contaminate syringes, needles, pipettes, and counting dishes and that the radioactivity might be removed by washing only to a limited extent prompted the investigation of this problem. A standard solution of plasma was prepared by adding sufficient iodinated radioactive human plasma so that 1 ml. of the material gave about 4,000 counts per minute. This solution was used to fill pipettes and syringes. Next, the material was allowed to run out of the pipettes, which were allowed to stand about ten minutes, and then washed in the various manners described (Table IV). The syringe was treated in a similar manner, but was washed with a detergent and water in a manner comparable to that which it would be subjected to in a central supply room of a hospital. After the washing, the glassware was dried and filled to the calibration mark with water. The water was transferred to a counting dish and allowed to dry, then counted. In all cases the amount of radioactivity removed from the glassware after washing was negligible. The counts of the wash solution were the same as the background counts. The glassware itself, after being washed, was placed close to the Geiger tube and showed no increase in counting rate, indicating that there was no I^{131} bound to the glass. To determine whether the same washing procedure might be effective in removing larger amounts of I^{131}-labeled protein from syringes, a 1 ml. tuberculin syringe which was used to withdraw the tagged albumin from the stock bottle (130 mc./per cubic centimeter) was rinsed successively with saline, water, soap solution, and distilled water. No brush was used in the washings. The syringe was then filled with distilled water, and after ten minutes 1 ml. was transferred to a counting dish. The dish was dried and counted. The washing from the syringe gave 44.8 counts per minute, while an empty dish (background) gave 24.4 counts per minute. This simple washing procedure apparently was not sufficient to remove all the activity from the

syringe. Thus, it would appear that human albumin tagged with radioactive iodine is readily washed from glassware, providing its activity is moderate and no elaborate precautions are needed to render the glassware free of radioactivity. For very active albumin, e.g., the stock solution, washings must be more vigorous to free the glassware of all activity.

SUMMARY AND CONCLUSIONS

Some of the technical problems involved in the determination of plasma volume using serum albumin tagged with I^{131} have been studied in an attempt to obtain better control over the method and thus reduce the variation. The error due to pipetting the plasma-containing radioactive material is within the counting error. An important factor to be considered is the variation in the structure of the counting dishes, which must have perfectly flat bottoms. After the plasma is pipetted into them, the dishes must be allowed to dry on a level surface. If these cautions are not observed, one may obtain dried films of protein possessing different thicknesses in different areas. The unevenness of the film can cause large discrepancies in the counts recorded. Providing one obtained uniformally dried layers of protein in the counting dishes, it is possible to count the sample as close to the Geiger-Müller tube as practical. The moving of the sample carrier closer to the tube will allow a much more rapid count without increasing the variation of the procedure. The glassware used in carrying out a determination with human plasma tagged with I^{131} can be washed in any of the usual manners employed in the chemical laboratory to render them free of radioactive contamination.

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STUDIES IN NONPROTEIN NITROGEN

III. Evaluation of Methods Measuring Creatinine

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THE original Folin-Wu method of determining creatinine in blood and urine by means of the Jaffé reaction[1] has undergone many modifications.[2] The color reaction with 3,5-dinitrobenzoate ("Bolliger reaction"), published independently from three laboratories in 1936,[3-5] has been much less popular, although claims for its greater specificity have been made.[5-7] The different procedures, likely to furnish different results, are dispersed through both old and recent periodicals and textbooks, and thus are available for indiscriminate use by laboratory workers.

In the course of an evaluation of the endogenous creatinine clearance as a measure of glomerular filtration, it was clearly desirable to select the most accurate and specific analytical method. Too, the use of such a method may conceivably enhance the diagnostic value of the serum creatinine level as an index of renal impairment.[8, 9] Hence, a study of representative modifications of the two types of color reaction was undertaken with respect to their accuracy, reliability, and practicality.

MATERIAL AND METHODS

Blood sera (or heparinized plasmas) and urines from persons with normal and with impaired renal function were analyzed by the methods described below. Similarly, the blood plasma of 30 rabbits was examined, some of which had been made uremic by poisoning with vinylamine.[10]

The Jaffé Reaction.—The method of Hare[11] (hereafter called H), which involves isolation of creatinine by adsorption on Lloyd's reagent, and the direct picrate method of Bonsnes and Taussky[12] (designated as BT) were studied as applied to protein-free serum filtrates and to diluted urines (deproteinized as serum, where necessary). The H method was carried out on three common types of filtrate: (1) using trichloracetic acid (Tc): 1 part of serum and 2 parts each of water and 20 per cent acid;[11, 13] (2) tungstic acid (Tu) in three different serum dilutions (described in Table I); (3) cadmium hydroxide (Cd): 1 part of serum, 3 parts of acid $CdSO_4$ solution (17.34 Gm. of $CdSO_4 \cdot 8\ H_2O$ + 84.55 ml. of 1N H_2SO_4 + distilled water to make 500 ml.) and 1 part of 1.1N NaOH.[11, 13, 15] All deproteinizing mixtures were allowed to stand for at least five minutes and then filtered through Whatman No. 2 paper. The H procedure was somewhat simplified by substituting conical for straight centrifuge tubes to permit pouring and thorough draining of the supernatant without disturbing the packed Lloyd's reagent; the latter was resuspended by means of glass rods in 4 ml. of alkaline picrate solution. The BT method was usually carried out in Tu filtrates. Cd filtrates gave closely similar results but presented no advantage; application to Tc filtrates (pH = 0.7) was attempted but gave unreliable results due to the low pH.*

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*This was not true in the case of acid Tu filtrates in which the pH was not low enough to alter the picrate color reaction.

TABLE I. TYPES OF TUNGSTIC ACID FILTRATES STUDIED

FINAL SERUM DILUTION IN FILTRATES	COMPONENTS			
	SERUM	DISTILLED H_2O	0.66N H_2SO_4	10% SODIUM TUNGSTATE*
	PROPORTIONS			
A–1:10	1	7	1	1[11, 12]
B–1:5	1	2	1	1
B′–1:5	1	3	$\frac{1}{2}$	$\frac{1}{2}$[6, 11, 13]
C–1:4	1	1	1	1

*The average pH of these filtrates is 6, but it is called "neutral" in the text in order to distinguish it from that of more acid filtrates (pH = 1.8) made by substituting 5 for 10 per cent sodium tungstate, following Brod and Sirota's formula for the 1:4 filtrate.[14] This substitution could not be applied in B′ because it resulted in a turbid filtrate due to incomplete protein precipitation.

The Bolliger Reaction.—When attempting to follow Langley's and Evans' procedure[5] and, subsequently, Fister's modification,[16] it was found that the time required for maximal color development varied between six and eight minutes, and that fading was noticeable within two to four minutes. The addition of 20 per cent sodium acetate did not significantly increase stability of color, in contrast to Roettger's contention.[7] Considerable manipulation, using varying amounts of the dinitrobenzoate reagent, sodium hydroxide, or sodium acetate, resulted in no improvement. Adjustment of pH of standards, blank, and test solutions, as recommended by Andes[17] and by Roettger,[7] was found to be necessary.

The following procedure was finally adopted as applied directly to Tc filtrates of serum or urine, prepared as outlined above: creatinine solutions ranging in concentration from 0.8 to 4.8 mg. per 100 ml. (made up with distilled water from a stock solution containing 1 mg. in 1 ml. of 0.1N HCl) and distilled water were substituted for the serum portion of the deproteinizing mixture to serve as colorimetric standards and reagent blank, respectively. To 5 ml. of each of these solutions and of filtrate, one drop of methyl red indicator (a 1 per cent solution in 95 per cent alcohol) and 0.8 ml. of 2.5N NaOH were added; thereafter, each sample was titrated with 0.3N NaOH to lemon yellow and made up with distilled water to a 7 ml. mark on the test tube, followed by 1.5 ml. of 10 per cent 3,5-dinitrobenzoate solution prepared as directed by Fister.[16*] The blank received 0.3 ml. of 2.5N NaOH, resulting in the rapid development of a stable orange color, and was then used to set the spectrophotometer at zero density with a wave length of 495 millimicra. Subsequently, the same amount of 2.5N NaOH was added to each standard and unknown, one at a time, and the ensuing progressive change in optical density, reflecting the "garnet-red" creatinine color reaction, was observed in each instance, and the peak reading recorded. This cumbersome procedure was necessary because of the color inconsistencies mentioned above. Values for the unknowns were obtained from a calibration graph which was found to follow Beer's law.

The procedure was also applied to Lloyd's adsorbate,[11] but had to be modified as follows: creatinine was eluted with 5 ml. of a dilute NaOH solution (1 part of 2.5N NaOH + 19 parts of H_2O). After centrifuging, 4 ml. of the supernatant was slightly acidified with 20 per cent trichloracetic acid, again using methyl red as indicator, then neutralized with 0.3N NaOH and made up to 5 ml. with distilled water. Addition of 3,5-dinitrobenzoate and of 2.5N NaOH followed and photometric readings were taken, as described above.

A Beckman Model DU spectrophotometer was used in all studies on human material, and a Coleman Universal spectrophotometer, Model 14, for those on rabbit plasma.

RESULTS

Comparison of Protein-free filtrates.—When the Hare method was applied to a Tc filtrate and, simultaneously, to either a Tu (B′ 1:5, Table I) or Cd filtrate made from the same human serum, the values derived from the Tc filtrate were always highest (Figs. 1 and 2). The Tu values constituted 90 to 96

*The reagent was carefully purified in accordance with Jansen's and associates' description[18]—but using 10 per cent sodium carbonate instead of hydroxide—to give a melting point of 206-207° C.

per cent, and the Cd values 88 to 93 per cent of the Tc values, the variations apparently not related to the serum creatinine level. Similarly, Tc filtrates, but not the other two types of filtrates, consistently permitted complete recovery of creatinine added to serum (Table II). This incompleteness of recovery, in the case of the Tu filtrate, was more pronounced in the "neutral" 1:10 than

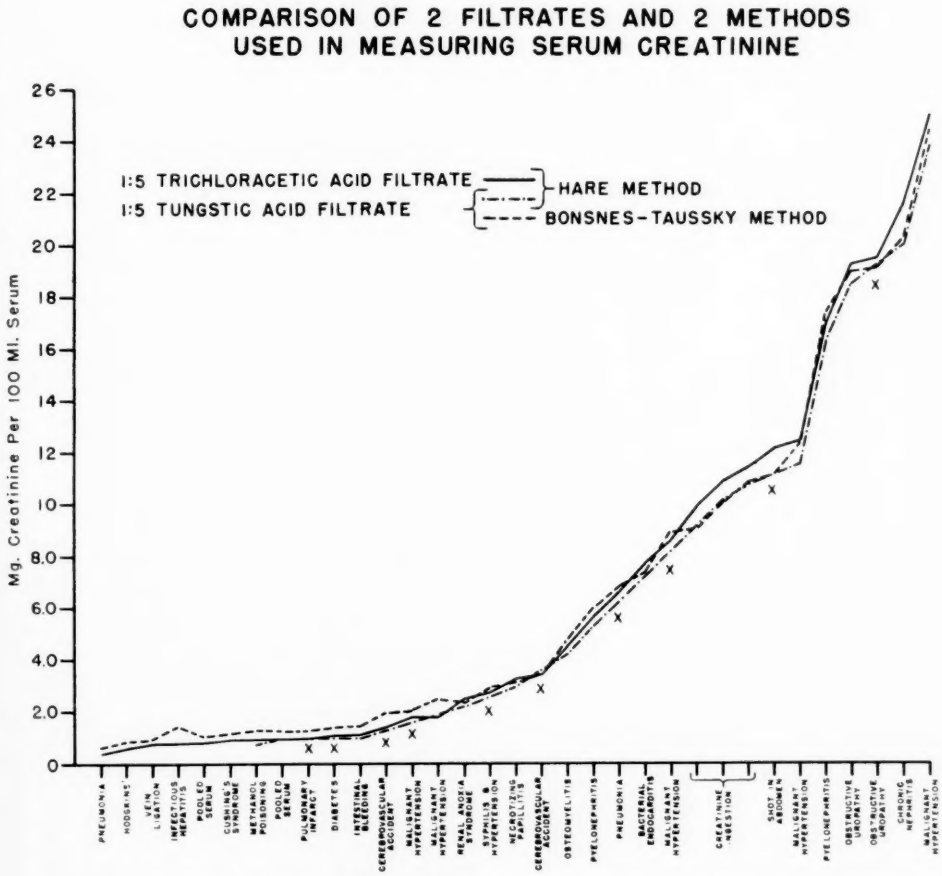

Fig. 1.—Creatinine values are plotted in order of magnitude, each value representing the mean of at least two replicate determinations (in this and subsequent figures). X-marks signify those instances in which H values on tungstic acid filtrates were not obtained and hence not plotted on the dot-dash line. The tungstic acid filtrates applying in this figure were "neutral" (B′, Table I)

At serum creatinine levels below 8 mg. per 100 ml., the standard deviation as determined from differences among replicates was, for the H method, 0.038 when applied to method to "neutral" 1:10 Tu filtrates. The standard deviation was 0.088 mg. per 100 ml. for Tc filtrates and 0.023 in Tu filtrates; it was 0.009 for the BT method. It increased to 0.069, 0.099 and 0.092 mg. per 100 ml., respectively, at higher levels.

1:5 (B′) dilution, but was not apparent when an acid 1:4 filtrate was employed. Recovery results were similar when the BT method was applied to these three Tu filtrates (Table II). The dinitrobenzoate (D) method was likewise tried on "neutral" 1:5 Tu filtrates (as recommended by Fister[16]) but, because of deficient recoveries, its application was eventually confined to Tc filtrates.

TABLE II. RECOVERY OF CREATININE ADDED TO SERUM

	TRICHLORACETIC ACID FILTRATE	TUNGSTIC ACID FILTRATE						CADMIUM FILTRATE
		1:10		1:5		1:4 (ACID)		
	H	H	BT	H	BT	H	BT	H
ADDED MG./100 ML.	% CREATININE RECOVERED							
0.8	101.8[5] (97.5-111.3)							
1.6	101.5[5] (99.3-102.5)	89.6[3] (85.1-93.1)	88.0[3] (84.4-92.5)	95.4[3] (94.1-96.6)	97.8[4] (91.3-105.9)	99.9[2] (99.0-100.9)	102.9[5] (95.5-109.9)	92.7[3] (88.0-98.5)
3.2	101.2[5] (97.6-104.7)	92.0[3] (87.5-94.7)	90.9[3] (84.3-98.2)	95.2[3] (93.8-96.6)	97.0[4] (95.0-99.9)	99.7[2] (98.3-101.2)	99.4[3] (94.6-106.0)	93.6[2] (92.4-95.0)
4.8		91.0[3] (86.7-96.5)	89.7[3] (83.3-96.2)	92.9[3] (89.8-95.0)	96.9[4] (93.5-98.4)	98.9[2] (97.5-100.3)	96.9[2] (95.7- 98.0)	93.1[2] (92.6-93.6)

Average per cent recoveries are expressed by single values, the range by values in brackets; the number of recovery experiments performed is denoted by indices. All analyses on which these percentage calculations are based were run in duplicates. The standard deviation as determined from differences among replicates was 0.027 mg. per 100 ml. for the Hare (H) method and 0.016 for the Bonsnes-Taussky (BT) method.

Since the acid 1:4 Tu filtrate gave better yields than the higher but "neutral" filtrate dilutions, the acid 1:10 and 1:5 and the "neutral" 1:4 dilutions (Table I) were studied. It is apparent from Tables III and IV that acid

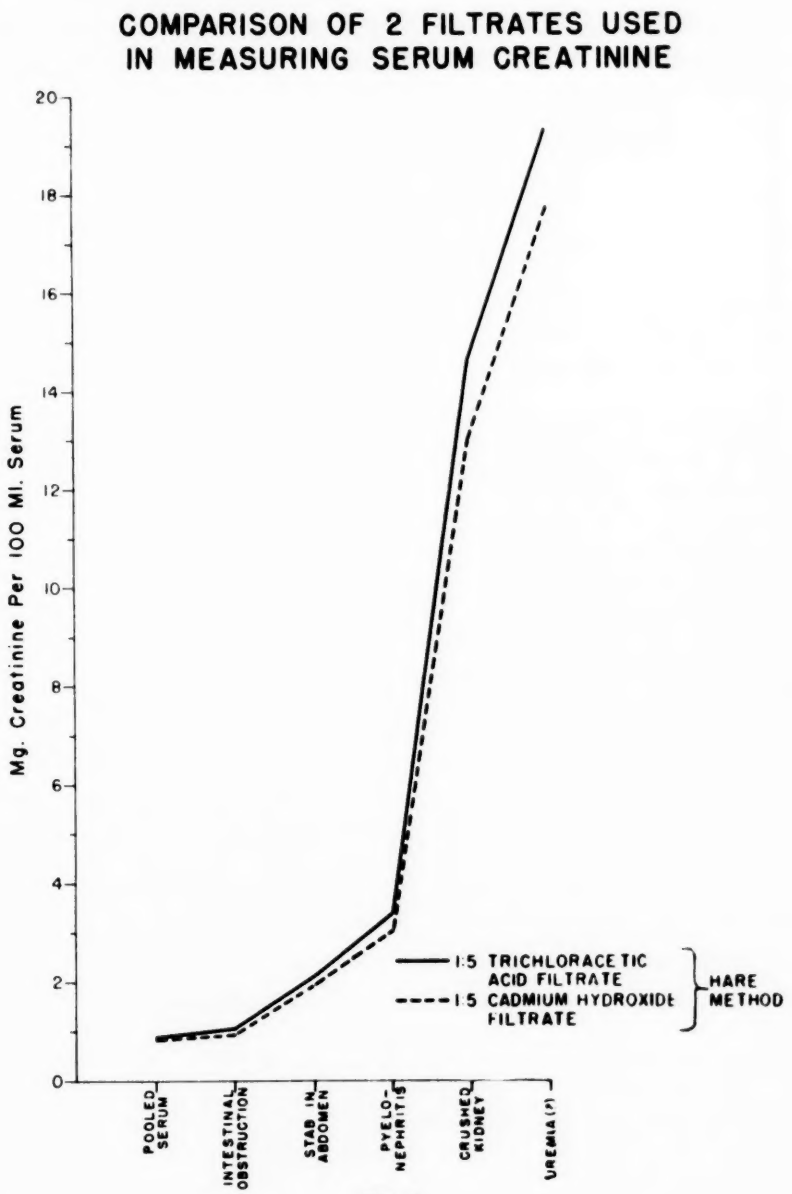

Fig. 2.

Tu filtrates from any of the three serum dilutions permitted a creatinine yield comparable with that of Te filtrates, while the corresponding "neutral" Tu filtrates gave consistently deficient results.

TABLE III. SERUM CREATININE VALUES AS YIELDED BY ACID AND NEUTRAL TUNGSTIC AND BY TRICHLORACETIC ACID FILTRATES (HARE METHOD)

NO. OF SPECIMEN	TC 1:5	SERUM FILTRATES TU ACID 1:10	ACID 1:5	ACID 1:4	NEUTRAL 1:10	NEUTRAL 1:5	NEUTRAL 1:4
		MG. PER 100 ML. SERUM*					
1	0.67			0.67			0.67
2	0.73			0.76			0.72
3	0.74		0.77	0.75		0.62	0.60
4	0.87			0.91			0.85
5	0.93			0.97			0.91
6	1.35	1.47			1.31		
7	1.68		1.72	1.68		1.44	1.36
8	2.53			2.54			2.39
9	10.31			10.30			9.83
10	11.06	10.84			9.54		

Tc = trichloracetic acid.
Tu = tungstic acid.
* = means of duplicate determinations.

Comparison of Methods.—The BT method gave significantly* higher values than the H method when both were applied to aliquots of the same Tu filtrates from human sera (Fig. 1). The difference ranged between about 18 and 31 per cent of the BT values at normal serum creatinine concentrations, but was less pronounced at increased levels. When the comparison was made with H values derived from Tc filtrates, the differences between BT and H values were smaller, though still statistically significant* up to a creatinine level of about 6 mg. per 100 ml. Beyond that level, the differences were not significant* and the relationship was, at times, even reversed, that is, H (Tc) values slightly exceeded the BT (Tu) values (−9 to + 7 per cent). The same phenomenon was demonstrated with rabbit plasmas (Fig. 3).

Also on analysis of urine the BT values were, as a rule, higher (up to 10.9 per cent) than the H values, although, in the majority of instances, the differences lacked statistical significance.* There were only five exceptions to that rule in the series of 39 protein-negative urines tested (Fig. 4), and four exceptions among 36 protein-containing urines (Fig. 5).

The D method showed a behavior quite similar to that of the BT method in that the D values, as derived from Tc serum filtrates, were always distinctly higher than the corresponding H values (Fig. 6). When applied to Lloyd's

TABLE IV. RECOVERY OF CREATININE ADDED TO SERUM

CREATININE ADDED MG./100 ML.	1:5 TUNGSTIC ACID FILTRATE* ACID H	ACID BT	NEUTRAL H	NEUTRAL BT
	% RECOVERED			
1.25	96.8	99.2	80.4	87.6
2.50	97.6	104.6	80.8	89.4
5.00	98.8	103.6	80.8	88.6

H = Hare method.
BT = Bonsnes-Taussky method.
*B, Table I.

*Statistical significance was determined at the 0.05 probability level.

eluate, the D values approximated the H values. In three urines, results of the D method did not differ materially from the BT values obtained concurrently.

COMMENT

Analysis of blood in this study was restricted to serum and plasma because of the fact, known since 1917,[19] that the red cells contain a large amount of noncreatinine chromogenic (NC) material.[20] The influence of the type of

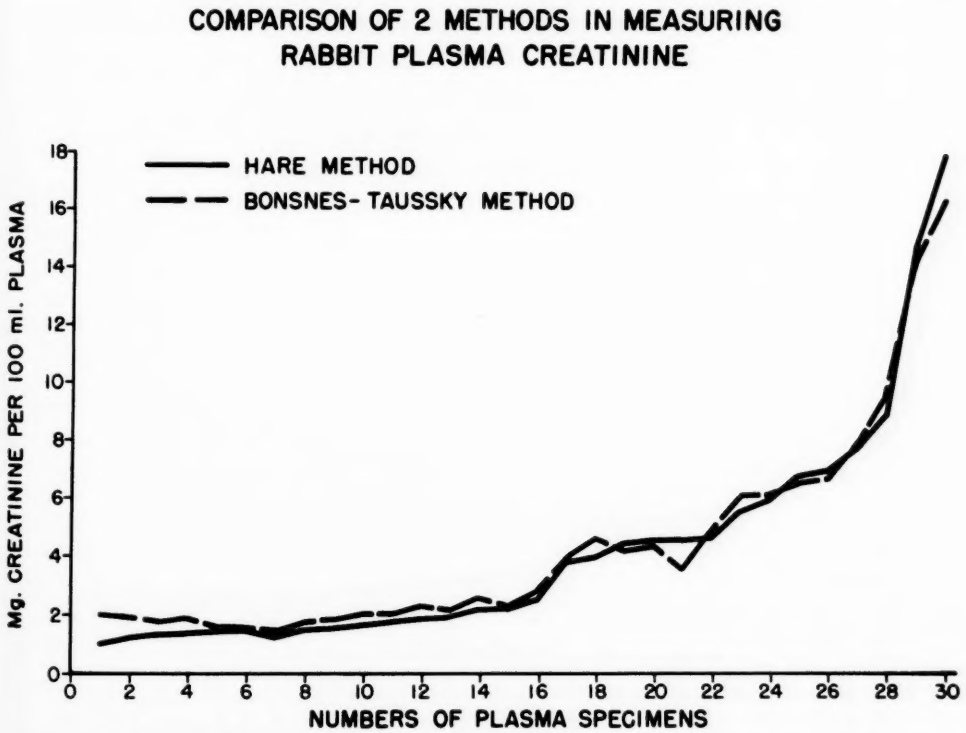

Fig. 3.—Note that the Hare method was applied to Tc filtrates and the Bonsnes-Taussky method to "neutral" 1:10 Tu filtrates. The standard deviation was 0.088 mg. per 100 ml. for the Hare method and 0.152 for the Bonsnes-Taussky method.

filtrate on the accuracy of serum creatinine values has not been apparent before, except for sporadic observations by Smith and co-workers,[13] and by Camara and associates,[21] indicating incomplete creatinine recovery from Cd and Tu filtrates, respectively. The latter workers noticed better recoveries from 1:10 (88 per cent) than from 1:4 (78 per cent) Tu filtrates (presumably of the conventional "neutral" type), attributing the difference to adsorption of more creatinine to the denser protein precipitate. Miller and Miller, in an attempt to check these findings,[22] obtained 3 to 13 per cent higher values from 1:10 than from 1:5 "neutral" Tu filtrates of six normal sera. Hare[11] seems to use interchangeably Tc, "neutral" Tu, and Cd filtrates, all constituting fivefold serum dilutions. Our data indicate that the creatinine yield from all the Cd and "neutral" Tu filtrates studies is deficient, with minor variations in degree among the three Tu filtrate dilutions used; whereas the yield is complete from acid

Tu filtrates (made by substituting 5 per cent tungstate for the usual 10 per cent solution [Table I]). This is not surprising if one remembers that deproteinization with bivalent metals in alkaline medium, as with $Zn(OH)_2$[23] and $Cd(OH)_2$,[15] has actually been designed in order to eliminate from the filtrate nitrogenous substances which would interfere with precise glucose analysis.

The differences between BT or D values, respectively, and H values as obtained from the same serum filtrates presumably represent NC material. Our range of 18 to 31 per cent of the total chromogen (BT) values for this fraction

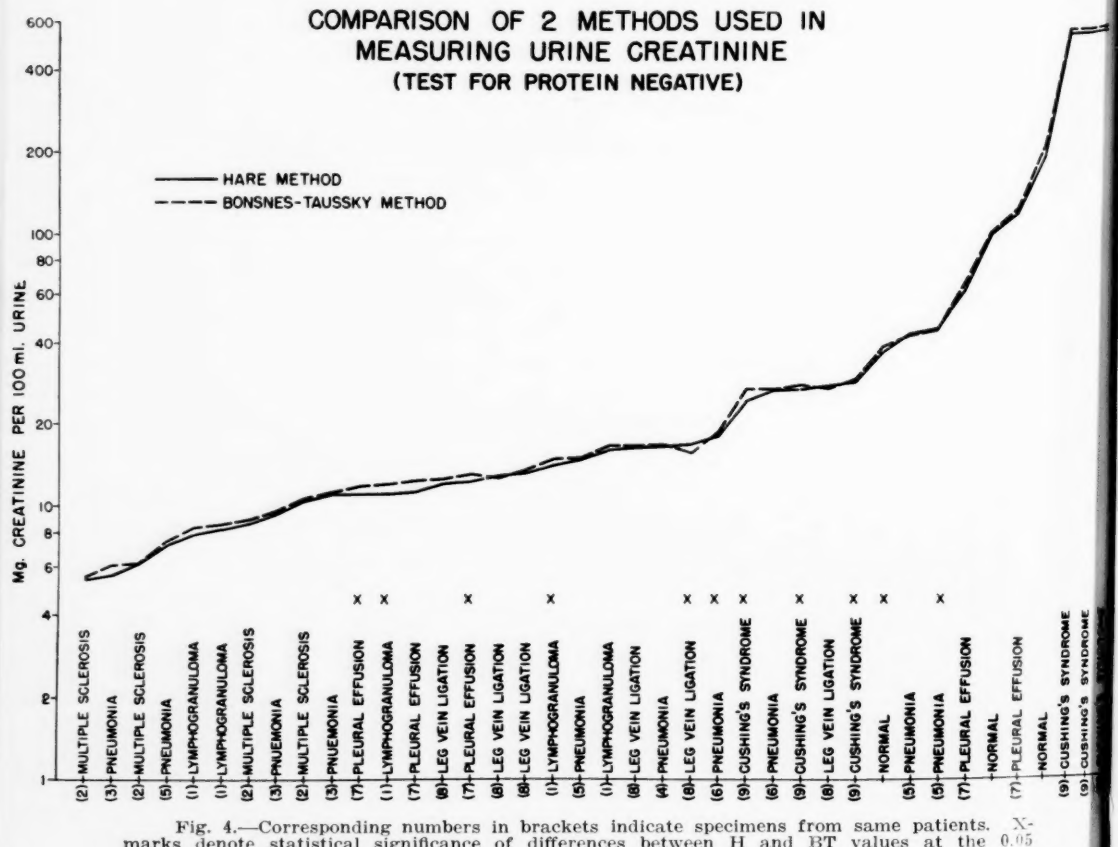

Fig. 4.—Corresponding numbers in brackets indicate specimens from same patients. X-marks denote statistical significance of differences between H and BT values at the 0.05 probability level (the test for significance was not applied above 45 mg. per 100 ml. because of the paucity of high values).

The over-all standard deviation for the H method was 0.27, for the BT method, 0.17 mg. per 100 ml.

at normal serum creatinine levels is distinct from the range of 0 to 19 per cent found by Miller and Dubos[20] by means of their enzymatic method. In azotemic sera, these authors found an NC portion of up to 31 per cent, in contrast to the low range of 0 to 12 per cent encountered by us. However, NC fractions calculated from results of a recent comparison by Miller and Miller of the BT, enzymatic, and adsorption methods[22] closely conform to ours. They range, for the adsorption (H) method, between 18 and 34 per cent of the BT values at

serum creatinine levels below 1 mg. per 100 ml., and between 0.5 and 11 per cent in sera, with concentrations greater than 2 mg. per 100 milliliter. Corresponding NC values for the enzymatic method are 13 to 28 and 4 to 14 per cent, respectively. Thus, the data of these authors and ours are in general agreement with the statement by Hare and Hare that in chronic renal insufficiency, the serum creatinine constitutes 90 per cent or more of the total chromogen.[24*] The results of Miller and Miller's studies with the specific enzymatic method, together with those of our recovery experiments, tend to confirm the H method as measuring "all the creatinine and only creatinine" in blood.[11]

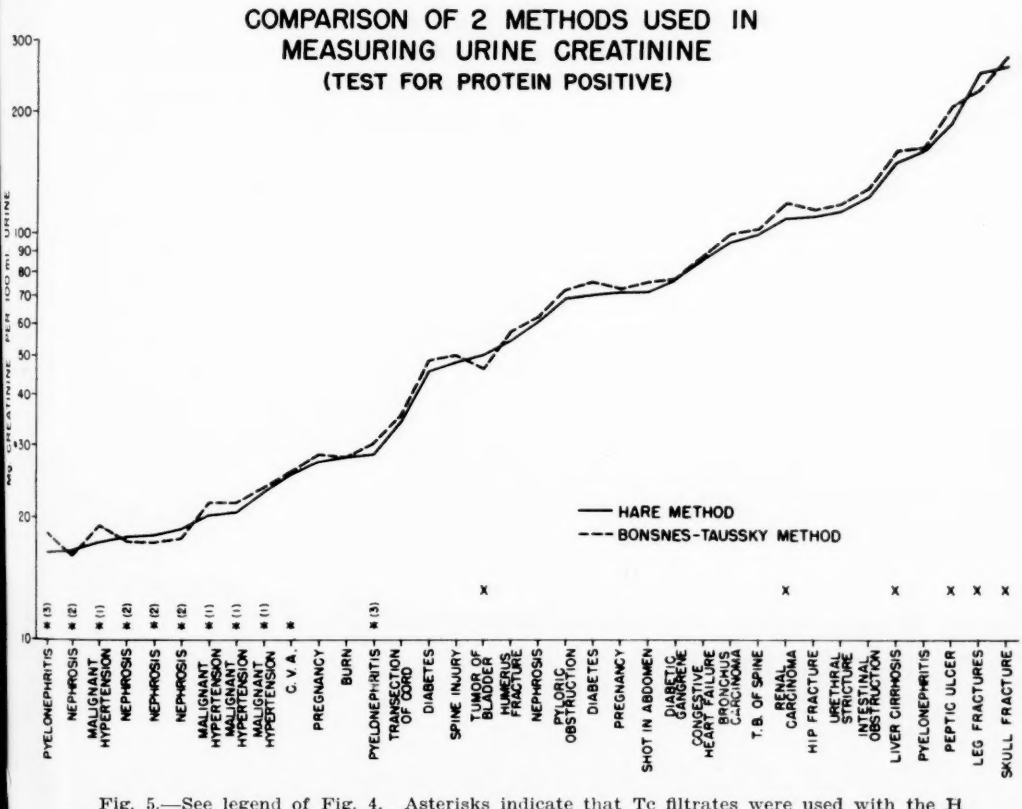

Fig. 5.—See legend of Fig. 4. Asterisks indicate that Tc filtrates were used with the H method and "neutral" Tu filtrates with the BT method. In all other instances, both the H and BT methods were applied to acid Tu filtrates.

The over-all standard deviation for the H method was 1.16; for the BT method, 1.58 mg. per 100 ml.

The BT method, if applied to "neutral" 1:5 Tu filtrates of azotemic sera, resulting in a 2 to 8 per cent loss of creatinine, furnishes values which closely approximate the actual serum creatinine content. This "fortunate combination of errors"—so called by Camara and associates [21]—is, at normal serum creatinine concentrations, far from being adequate in rectifying BT values, as shown in Table II and Fig. 1. The inaccuracy of the BT method is further augmented

*Lauson recently demonstrated partial suppression of the picrate color contributed by NC substances in the presence of increased creatinine concentrations.[25]

by the progressive increase in the optical density of the picrate color reaction in serum filtrates, an observation recently stressed by Hare[11, 24] and denied by Bucht[26] but verified by us on numerous occasions. This change in optical density, requiring accurate timing of colorimetric readings, is probably the reason

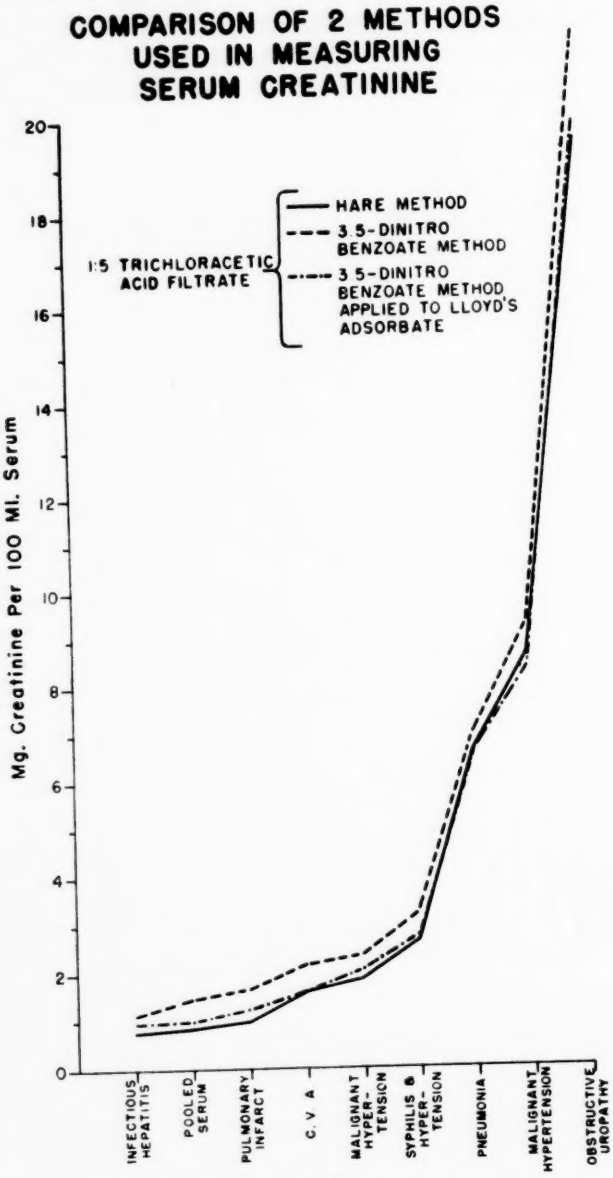

Fig. 6.

why the experimental error of this method can be greater than that of the H method, which involves far more manipulation (see Figs. 3 and 5).

Instability of color—though of a different nature, as pointed out initially—as well as lack of specificity also characterize the D method. The latter

quality was indeed disappointing in view of optimistic statements by earlier workers.[5-7] The NC fractions of corresponding D and BT values are of the same magnitude, even though they must differ chemically; e.g., glucose does not react with dinitrobenzoate, but may contribute to the picrate color.[4, 7, 25*] The cumbersome manipulation inherent in purifying the reagent and the complicated analytical procedure would seem to deter use of the D method altogether.

The differences between H and BT values encountered in the urines analyzed by us are quite in agreement with pertinent data by Miller and Dubos[20]: in six of thirteen (normal and abnormal) urines tested with their enzymatic method, creatinine constituted 100 per cent of all the Jaffé-reactive material; in seven it ranged from 90 to 99 per cent. In our study, fifty-eight of seventy-five urines showed no significant† difference between H and BT values; in thirteen, creatinine—as represented by the H values—constituted 90 to 97 per cent of the total chromogen (i.e., BT values), while in four, it seemingly amounted to more than 100 per cent—a finding which may be due to an unusually large technical error. This error, of course, is ordinarily greater in urine than in serum analysis, owing to the added manipulation involved in diluting the former for the purpose of bringing colorimetric readings within optimal range. Hare[11] found from 0 to 10 per cent of NC material in urine, but gave no details. Hence, it would appear that results of the direct picrate method are sufficiently accurate for most purposes. The continued clinical use of this procedure in serum analysis may be justifiable in view of the simplicity of operation and enhanced accuracy in the presence of renal failure. For this same reason, namely, because the NC fraction is rendered insignificant by an increased serum creatinine level, the validity of many published data concerning exogenous creatinine clearances need not be questioned.[2, 13] Results of estimation of the endogenous clearance, however, based on a direct picrate method,[2, 3, 9, 14, 21, 26, 28] must be regarded with due reservation. For such investigations, as well as for exact studies of the serum creatinine content, the adsorption-elution method,[11, 24] even though more time-consuming, is the method of choice, coupled with a deproteinizing procedure which insures complete creatinine filtration.

SUMMARY AND CONCLUSIONS

Of three common techniques of deproteinization tested, only the one using trichloracetic acid permitted complete creatinine filtration. Tungstic acid was equally satisfactory under conditions resulting in a distinctly acid filtrate.

The color reaction of 3,5-dinitrobenzoic acid with creatinine is no more specific than that of picric acid and it is much less practical. The direct picrate method of Bonsnes and Taussky has an inherent positive error of 18 to 31 per cent at normal serum creatinine concentrations, and is therefore too insensitive to detect slight alterations in concentration; its accuracy improves with

*Pyruvic acid was recently shown to constitute a significant portion of the total picrate chromogen but was not tested against dinitrobenzoate.[27] The proposed method of oxidizing pyruvic acid for the purpose of measuring the true creatinine will require careful checking before acceptance, particularly as the normal values were unusually low (0.3 – 0.6 mg. per 100 ml.).

†See footnote page 328.

increased serum creatinine contents and, when applied to urine, approaches that of the adsorption-elution method. The latter, though more cumbersome, excels in specificity and accuracy.

Statistical analyses were carried out by Mr. Myron J. Willis, Communicable Disease Center.

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A METHOD FOR BLEEDING MONKEYS USING AN INTRA-AORTIC POLYETHYLENE CATHETER

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IN ORDER to obtain the greatest possible amount of serum from monkeys vaccinated with poliomyelitis virus, several methods of bleeding have been tried. These include cardiac puncture, cannulation of the femoral and carotid arteries, and incision of the jugular vessels to permit the flow of blood into a sterile beaker. The method that yields the greatest amount of blood with least difficulty is passage of a polyethylene catheter (Clay-Adams) through a femoral artery into the thoracic aorta. This method is presented in detail with the thought that it may be useful for other purposes and for other animals.

METHOD

The monkey is lightly anesthetized with intravenous Pentothal Sodium and restrained on his back on a dissecting board. The femoral region is shaved and prepared with iodine and alcohol. An incision three to five centimeters in length is made from the inguinal ligament down over Scarpa's triangle. The femoral artery is freed by blunt dissection (Fig. 1) and two silk threads are passed under the artery. An assistant makes a loose half knot in the proximal thread and pulls the thread taut enough to obstruct flow through the artery. The operator steadies the artery with the distal thread, and with fine scissors makes a nick in the arterial wall between the threads (Fig. 2). The beveled end of a twenty-two inch length of polyethylene tubing, of a diameter to fit a No. 18 needle, is passed into the artery for a distance of eight to ten inches (Fig. 3). The half knot in the proximal thread is tightened to secure the catheter in place. Blood is collected in a 250 ml. centrifuge bottle (Fig. 4). The entire procedure takes only a few minutes, permitting as many as eight monkeys to be bled simultaneously.

The amount of blood that can be collected without killing the monkey is variable. As much as 200 ml. have been obtained at a single bleeding from monkeys weighing ten to twelve pounds. Although it is not intended to bleed the monkeys to the point of surgical shock, this sometimes is done. The shock state usually can be reversed by releasing the limb restraints, which trap considerable blood; and by putting the monkey in a head low position. Physiologic saline, warmed to 37° C., can be injected through the catheter in an effort to restore the circulating blood volume. This seems to have at least a temporary restoration effect on monkeys that have been bled excessively. To terminate the bleeding the catheter is pulled out and the artery is ligated. The wound is washed with a copious amount of warm saline and the edges are approximated with interrupted catgut sutures.

During the recovery period the animals are given prophylactic penicillin injections and fed a diet enriched with protein and iron. The monkeys usually are active within a day, and after two or three weeks they are vigorous enough for another bleeding. If the first bleedings are taken distally in the femoral artery, it is possible to bleed again from that artery using a more proximal site.

DISCUSSION

Polyethylene tubing has been found useful to the clinician for replacement transfusions[1] and intravenous fluid therapy because it is relatively nonirritating,

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it is not readily obstructed with clotted blood, and it can be secured in a blood vessel with little danger of being dislodged. These same reasons make the tubing ideal for use in the laboratory, as described here for the bleeding of monkeys. It would seem to be equally useful for the bleeding and administration of fluids to other animals, such as dogs and cats, and for the kymographic recording of blood pressures. Advantages over conventional cannula and stylet consist largely in ease and speed of insertion and absence of obstruction due to blood clots.

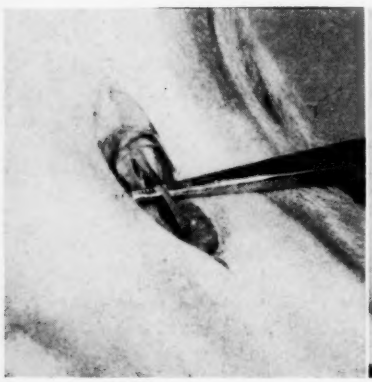

Fig. 1.

Fig. 2.

Fig. 3.

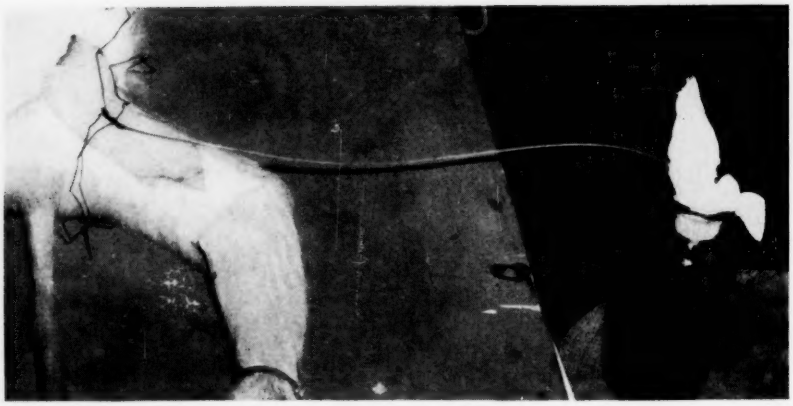

Fig. 4.

SUMMARY

A technique for bleeding monkeys using a polyethylene catheter in the femoral artery is described. The technique results in a much higher yield of blood than the cannula and stylet formerly used.

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